The cellulase system of Cephalosporium acremonium Corda.

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THE CELLULASE SYSTEM OF CEPHALOSPORIUM ACREMONIUM CORDA.

A thesis submitted by
DAVID WRIGHT
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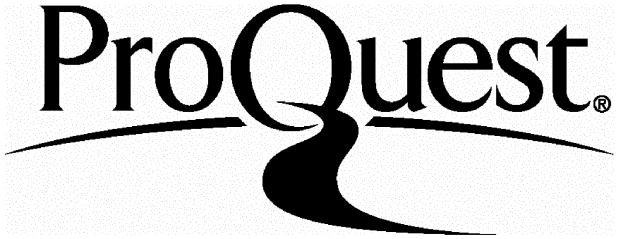
.....D. Wright.....



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SUMMARY

1) Growth of Cephalosporium acremonium was supported by a variety of carbon sources ranging in complexity from glucose to native cotton. Growth on carboxymethyl cellulose was however limited, believed to be due to the metabolic inability of the organism to utilize the substituted products of attack. Although viable cultures of C.acremonium were capable of rapidly solubilizing cotton boll fibres which had never been allowed to dehydrate, growth on dried cotton fibres was characterized by the inability of the organism to produce complete fibre solubilization.

2) Substrates possessing β-1,4-glycosidic bonds produced enhanced titres of cellulolytic enzymes but were not essential for enzyme induction.

3) Cell-free extracts obtained from such cultures possessed appreciable hydrolytic activity towards cellobiose, CMC and acid swollen cellulose, but were considerably less active on native cotton fibres.

4) Although the presence of cell-bound cellulase enzymes was established, further investigation indicated these components to be similar in type, number and mode of action to those isolated from culture filtrates.

5) Fractionation of cell-free extracts resolved the cellulase system into three distinct enzymic components whose actions overlapped, and which differed mainly in molecular weight and in their relative affinity for substrates of varying complexity. These consisted of (a) a β-glucosidase possessing a molecular weight in excess of 200,000 and active mainly on soluble low DP substrates; (b) a CMC-ase with a molecular weight of

approximately 45,000, active on CMC and the higher DP oligosaccharides. Although this component exhibited no hydrolytic activity towards insoluble cellulosic substrates, the enzyme was found to possess slight S-factor activity and to be capable of producing a detectable decrease in the tensile strength of cotton. (c) Cx-cellulase, an enzyme with a molecular weight of about 11,200 which exhibited little substrate specificity. This enzyme was responsible for the activity of the organism towards native cotton, but was also capable of producing extensive hydrolysis and depolymerization of less structurally complex substrates and oligosaccharides.

6) All three components were found to be complexed with carbohydrate.

7) Viscometric studies indicated the action of the depolymerizing CMC-ase and Cx-cellulase components to be endoenzymic.

8) Enzyme/substrate affinity determinations revealed a decrease in affinity as the structural complexity of the substrate increased. These results suggested the existence of only a limited number of sites in native cotton fibres accessible or susceptible to attack by the Cx-cellulase.

9) Evidence of increased crystallinity in the residual short fibres produced from native cotton by cultures of C.acremonium suggested that such sites consisted of essentially amorphous cellulose. Evidence in support of this theory was provided by the observation that amorphous undried cotton boll fibres were completely solubilized by this organism.

10) The extensive but incomplete degradation of native cotton by viable cultures of C,acremonium was acheived without the aid of a C1 component. Solubilization of the residual short fibres could be rapidly completed by a culture of T.viride.

INTRODUCTION

CELLULOSE is the most abundant naturally occurring organic compound, comprising one-third to one-half of the bulk of all dry vegetation. As the world's most plentiful renewable resource its decomposition plays a vital role in the carbon cycle, replenishing the atmospheric carbon dioxide necessary for the continuation of photosynthetic processes. It is possible that should the natural decomposition of cellulose ever cease, this atmospheric carbon dioxide pool could be maintained at a satisfactory level for some time by the release of carbon dioxide dissolved in the oceans and from carbonates in marine sediments. Dead vegetation unable to rot would however quickly accumulate and suppress fresh plant growth with disastrous consequences for a growing world population.

The decomposition of cellulose is brought about mainly by enzymes of microbial origin, although the organisms responsible for the production of these enzymes may be associated with an animal symbiont as in the case of ruminants. Cellulolytic micro-organisms are however mostly free-living and are widespread in nature, this distribution being a great biological blessing and at the same time a serious limitation to the usefulness of all cellulosic materials. Although most vegetation is unused by man or animals, enormous quantities are present in manufactured products such as textiles, paper and building materials. As cellulolytic micro-organisms are unable to distinguish useful cellulosic materials from plant debris, man has found it necessary to invest huge sums of money in attempts to prevent the degradation of his more essential cellulosic commodities, the main incentive behind many early studies on the mechanism of degradation being

the production of effective inhibitory treatments. While the preservation of certain cellulosic materials is still of considerable economic importance, it is perhaps paradoxical that the growing use of synthetic fibres and plastics and the pressing problems of environmental pollution and world famine are currently placing increased emphasis on facilitating the breakdown of cellulose debris. The population explosion is straining already overloaded waste disposal schemes in which cellulosic material is one of the most difficult components to be dealt with successfully, and is creating tremendous demands on world food supplies. These demands have aroused considerable interest in the production of foodstuffs from unconventional sources, and it is surprizing that cellulose has as yet received so little attention in this respect, being plentiful and widely distributed throughout developed and underdeveloped countries.

In working towards the prevention of disintegration of useful cellulosic materials and towards the enhancement of decomposition of plant wastes, the enzymic process of the biological degradation of cellulose has to be clarified. Despite the diligent efforts of many able investigators since the time of DeBary (1886), present knowledge of this process remains far from complete; indeed when attempting to assess the voluminous literature which has accumulated on the subject over this period one is impressed by the still rather confused state of the art. This is undoubtedly due to organism-substrate relationship problems specific to cellulose and cellulolytic micro-organisms, and for which satisfactory explanations are even now only in the early stages of evolution. As it is now apparent that the structural complexity of the cellulose substrate is one of the main factors affecting its susceptibility to enzymic

attack, any review of the progress made in cellulase research must be made in the light of current theories concerning the fine structure of cellulose.

In 1897, the discovery by the Buchners that a cell free extract of yeast was capable of fermenting sugars to ethanol and carbon dioxide paved the way for the tremendous advances in general enzymology made since that date. The elucidation of basic enzyme mechanisms has been considerably simplified by the use of such cell-free extracts, and their preparation from almost every type of plant, animal and microbial cell has for many years been a routine biochemical procedure. It is hardly surprizing therefore that the characterization and mode of action of cellulolytic enzymes have remained unresolved when it is realized that until 1963 no cell-free extracts possessing significant activity towards native untreated cellulosic substrates had been prepared. Early investigations soon revealed that although growing cultures of certain cellulolytic micro-organisms rapidly degraded all forms of unmodified cellulose, filtrates prepared from even the most active of these cultures exhibited little or no activity towards the same substrates despite the fact that cellulolysis must by its nature be an extracellular process. Selby (1963) quoted the example of Myrothecium verrucaria of which whole cultures were capable of reducing the strength of a cotton yarn to zero in 4-5 days, whereas cell-free extracts prepared from the same cultures would at best reduce the strength by only 30% even after incubation at optimum temperature and pH for several weeks. This anomaly, coupled with other early microscopical observations that the dissolution of wood fibres by Hymenomycetous fungi (Hubert, 1924) and by the so called soft-rot fungi (Bailey &

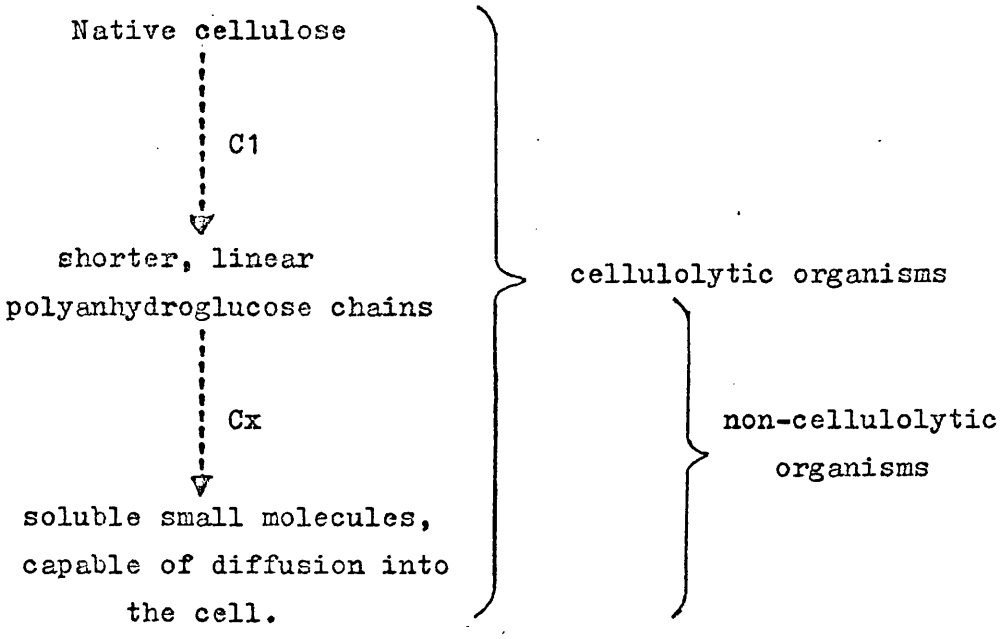
Vestal, 1937; Duncan, 1960) was highly localized to the penetrating hyphal strands led to the general belief that the degradation of such native fibres occurred only in close proximity to the cellulolytic micro-organisms, (Gascoigne & Gascoigne, 1960). Although this presumably implied the action of cell-bound enzyme components, no detailed investigations into the possible existence of such components were apparently made. Selby (1963) however, believing that the organism could perhaps produce local high concentrations of enzyme on the surface of the substrate investigated and subsequently disproved this theory. Evidence in support of enzyme action in areas remote from the organism was however provided by Cowling (1961) and by the electron microscopy studies of Meier (1955). Cowling showed that the boreholes formed in wood blocks by cellulolytic fungi could account for only 6% of the total loss in weight of the wood measured during decay. This observation suggested that voids incapable of resolution by light microscopy were being created during attack, a theory in agreement with the earlier work of Meier (1955) who showed that brown-rot fungi produced areas of low electron density within the cell walls of wood fibres. These areas were presumed to be regions from which carbohydrates had been removed, leaving a lattice-work of lignin-rich material.

Despite these and other <u>in vivo</u> observations on the destruction of wood by various fungi (Hubert, 1924; Scheffer, 1936; Waterman & Hansbrough, 1957), cell-free extracts prepared from such organisms remained essentially inactive against native cellulose in the form of either cotton or wood fibres. Such extracts were however found to be quite effective in degrading cellulosic substrates which had been rendered more accessible to attack by exposure to

various forms of chemical pretreatment, the most simple of which was to swell the fibres in a concentrated solution of sodium hydroxide (Whitaker, 1953; Sison et al, 1958). If the alkali was then washed out with water and the swollen cellulose degraded without drying, all of its strength and some 30% of its weight could be rapidly lost (Selby, 1963). As a general rule the susceptibility of these substrates to enzymic attack was related to the extent of the pretreatment which they had received, an observation which led to the popular use of more highly degraded substrates such as amorphous particulate suspensions of cellulose (Norkrans, 1950a; Jermyn, 1952; Walseth, 1952; Basu & Whitaker, 1953; Whitaker, 1953; Tracey, 1955; Aitken et al, 1956; Sison et al, 1958). These suspensions were usually prepared by the prolonged action of concentrated inorganic acids, followed in many cases by mechanical dispersion treatments such as ball-milling(Reese et al, 1957; Saunders et al, 1948). In addition to being actively degraded by cell-free extracts they were also found to be much more convenient to handle than the original fibrous material (Blum & Stahl, 1952; Halliwell, 1961), features which suggested as the next logical step the possible use of completely soluble cellulose derivatives as substrates. These derivatives, produced by chemical substitution of hydroxyl groups within the cellulose molecules had been widely used prior to this suggestion in the form of gels for pharmaceutical and other industrial purposes, the most common types being carboxymethyl cellulose (CMC), hydroxyethyl cellulose (HEC) and methyl cellulose. Evidence of their bio-degradability had in fact been noted by Woodward (1948) who isolated Aspergillus niger and A. flavus from a preparation of CMC which had liquefied on standing; by Freeman et al (1948) and Harry (1948) who from similar preparations

isolated bacterial cultures capable of liquefying fresh CMC, and by Siu et al (1949) who found that Myrothecium verrucaria successfully metabolized CMC and methyl cellulose. As expected, these substrates were indeed found to be even more rapidly hydrolysed by cell-free extracts than the treated fibrous and particulate celluloses (Blum & Stahl, 1952; Jermyn, 1952; Kitts & Underkofler, 1954; Aitken et al, 1956; Halliwell, 1957; Sison et al, 1958; Nisizawa & Hashimoto, 1959; Storvick & King, 1960), and in addition possessed the important advantage of being water soluble, a feature considerably simplifying elucidation of enzyme mechanisms by eliminating the problems of variable accessibility which occur when the substrate is insoluble. Such derivatives consequently became widely used to characterize cellulases. Work with soluble celluloses however furnished little information on the mode of attack of native cellulose and no explanation of the inability of cell-free extracts to degrade this highly ordered substrate. In 1950 however, Reese, Siu and Levinson described in some detail the ability of micro-organisms to utilize substituted celluloses as carbon sources, and observed that whereas numerous organisms were capable of growth on accessible or solubilized substrates, the ability to utilize native cellulose as sole carbon source was much more restricted. Reese and his co-workers attempted to explain these observations and the anomalous behaviour of cell-free extracts by postulating the existence of an enzyme termed C1 which they believed was able to split off linear chains of glucose residues from native cellulose. These chains which collectively form the cellulose structure would then be depolymerized by a different cellulase termed Cx. Those organisms unable to synthesize C1 would consequently be inactive on native cellulose, but would

be capable of hydrolysing modified substrates by virtue of their Cx component. These types were consequently classified as non-cellulolytic, the term cellulolytic being reserved for those organisms possessing both C1 and Cx enzymes, thus:



Culture filtrates from truly cellulolytic micro-organisms could therefore be presumed to be inactive against native cellulose because the C1 component was for some reason missing from the extracts as normally prepared.

Despite the widespread acceptance of the C1-Cx theory and the obvious importance of the C1 enzyme in natural degradation processes, it was in fact the Cx component which received most attention during subsequent years, resulting in the publication of numerous conflicting reports. A number of workers (Jermyn, 1952; Reese & Gilligan, 1953; Gilligan & Reese, 1954; Grimes, 1955; King, 1956; Miller & Blum, 1956; Hash, 1957; Norkrans, 1957a, 1957b; Hash & King, 1958; Miller, Blum & Hamilton, 1960; Nisizawa *et al*, 1962; Peterson & Porath, 1963; Peterson, Cowling & Porath, 1963; Logan &

Siehr, 1966; Patni & Rege, 1969) demonstrated that there were several enzymes all performing what Reese and his colleagues had described as Cx function which were separable by electrophoretic and chromatographic techniques, and which in most cases differed in the relative ease with which they degraded substrates of different chain lengths. Whitaker (1953) however concluded that a partially purified filtrate from Myrothecium verrucaria contained only a single enzyme, a theory supported by Kooiman et al (1953) who based their argument on the supposition that different enzymes acting on different substrates would be unlikely to show similar thermal stability patterns. Selby & Maitland (1965) subsequently produced good evidence however that the cellulase system of M. verrucaria is in fact heterogenous, the enzymic components differing in molecular size and in their relative activities towards different cellulosic substrates. In an effort to explain this apparent multiplicity of Cx components, Thomas & Whitaker (1958) suggested that there were in reality complexes formed between a single enzyme and several different polysaccharides, a theory supported by Van Haga (1958) and Miller & Birzgalis (1962) as a result of gel electrophoresis and column chromatography studies of M. verrucaria extracts. As however no assessment of activity towards native cellulose was made in any of the foregoing work, these observations were once again confined solely to the Cx system.

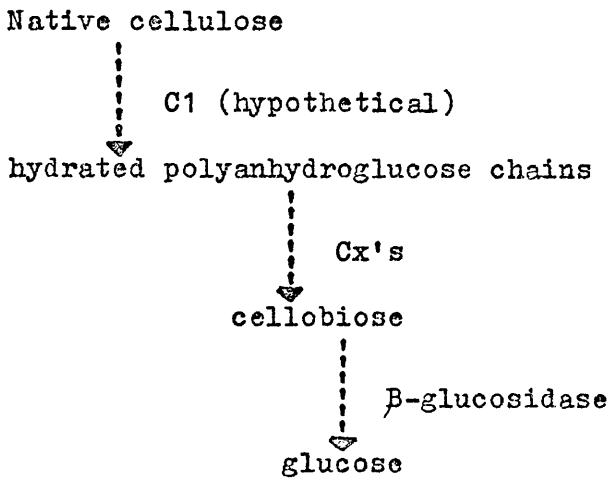
Observations on the mechanism of attack on modified celluloses by Cx components tended in general to be less conflicting. As a result of current studies, polysaccharases may be classified into two groups according to the mode of hydrolysis; exo- or endwise splitting, and endo- or random splitting enzymes. The latter

randomly hydrolyze any intramolecular bond whereas the former eliminate mono- or disaccharide units one at a time from the end of the chain. Although some evidence for exo-Cx cellulases has been published (Nisizawa & Kobayashi, 1953; Youatt & Jermyn, 1959; Gascoigne & Gascoigne, 1960; Storvick & King, 1960; Li, Flora & King, 1965; Okada et al, 1968; King & Vessal, 1969), the Cx enzymes of most micro-organisms investigated would seem to be of the random type (Whitaker, 1953; 1954; 1956; 1957; 1959; Gilligan & Reese, 1954; Reese et al, 1959; Hashimoto & Nisizawa, 1960; Shibata & Nisizawa, 1962), being characterized by their broad substrate specificity which differed only in the case of short chain oligosaccharides. It should be noted however that in contrast to the variety of experimental approaches available for testing random attack by enzymes, endwise degradation has usually been indicated by an isolated observation; namely the demonstration of a single sugar as the only hydrolytic product. The absence of intermediates is at best negative information and therefore less than convincing. In the case of soluble cellulose derivatives the approach has an even greater limitation in that even a true exoenzyme should yield intermediate products if only one or a few hydrolytic events follow each formation of an enzyme-substrate complex. In evaluating any report of exoenzymic activity therefore it must be borne in mind that purely qualitative analysis for intermediates and products cannot be expected to distinguish reliably between random and the two known types of endwise attack. More reliable experimental approaches to this problem such as the use of isotopically or chemically labelled substrates have tended in most cases to be cumbersome, although Reese (1969) has claimed to have developed a simple method for the estimation of exo-β-1,4

glucanase which is believed to be effective even in the presence of endoglucanase, this method being based on the ratio of activities of an extract in releasing glucose from cellotetraose and cellobiose. Because cellotetraose is much more rapidly hydrolysed than cellobiose by exo-β-1,4 glucanase, and much less rapidly hydrolysed than cellobiose by cellobiase (β-glucosidase), a high ratio would indicate a predominance of exoglucanase, and a low ratio a predominance of β-glucosidase. Although endoglucanases are usually regarded as acting at random on glucose polymers, Reese claimed that the presence of these enzymes would not markedly affect the ratio because of their relative inactivity towards terminal linkages. Phosphorylases which would however be capable of liberating glucose from cellobiose and cellotetraose were fortunately found to be absent from most extracellular extracts. Reese however made no reference to possible interference by glucotransferases, although it is likely that these enzymes would only markedly affect the overall reaction rate and not the final ratio of activities.

As originally postulated by Reese, Siu & Levinson (1950), the net result of Cx action whether endwise or random in nature is to liberate soluble sugars capable of diffusion into the microbial cell. Most reports have indicated that the endwise acting enzymes are capable of releasing only glucose (Reese, 1956; Li et al, 1965; Jurasek et al, 1967; King & Vessal, 1969) or cellobiose (Nisizawa & Kobayashi, 1953; McBee, 1948; Walseth, 1952; Reese, 1956; Ayers, 1958; Hulcher & King, 1958a; 1958b; Storvick & King 1960) as end products. Although the endo-Cx cellulases have been described as hydrolyzing any intramolecular bond, cellobiose which contains such a bond has frequently been found to occur both in the presence and absence of glucose as an end product of this type

of enzymic attack (Kooiman *et al*, 1953; Reese, 1956; Logan & Siehr, 1966). Nisizawa *et al*, (1963) demonstrated an endoenzyme obtained from *Irpex lacteus* to be capable of hydrolyzing a series of substrates from native cellulose to cellotriose to produce a mixture of glucose and cellobiose. This enzyme however became less active as the chain length or degree of polymerization (DP) of the substrate decreased, hydrolyzing cellotriose only slowly and cellobiose not at all. Cellobiose was however rapidly hydrolysed by another enzyme, β-glucosidase, to yield glucose. In the case of *Pseudomonas fluorescens* also investigated by these workers and in a species of *Alternaria* examined by Logan & Siehr (1966), β-glucosidase was presumably absent as cellobiose was not degraded. These results were in agreement with those obtained by King (1963) using a Cx cellulase preparation obtained from *Cellvibrio gilvus* which was also inactive on cellobiose and exhibited increased affinity for substrates of longer chain length. In view of the fact that glucose had been generally regarded to be the principal product of cellulose hydrolysis required by the growing cells as an energy source, β-glucosidase became accepted as a necessary addition to the original C1-Cx spectrum of enzymes. The scheme of Cowling (1963) can thus be diagramatically expressed as:



During early investigations β-glucosidase was often referred to as cellobiase, a term now seldom used with the realization that in most cases this enzyme is capable of hydrolysing not only cellobiose but also most short chain oligosaccharides upto cellohexaose (Reese, Maguire & Parish, 1968; King & Vessal,1969; Wood, 1971). These substrates have thus been found to be common to both β-glucosidase and Cx-cellulase, the former hydrolysing the smaller oligomers most rapidly and the latter the larger. β-glucosidase could consequently be regarded as a type of Cx-enzyme with a limited substrate range on the basis of these results. In contrast however to the more common types of cellulase complexes producing glucose as principal end product, the degradation of cellulose by Cellvibrio gilvus has been shown to result mainly in the formation of cellobiose and cellotriose (King & Vessal, 1969). Earlier studies (Hulcher & King, 1958) had indicated that cellobiose was in fact capable of being directly metabolized by undergoing intracellular phosphorolysis, with the subsequent demonstration (Schafer & King, 1965) that C.gilvus cells were capable of taking up not only cellobiose but all the members of the cellulose oligosaccharide family by an active transport system. The oligosaccharides were then apparently interconverted without loss of glucosyl bond energy by a glucotransferase situated in the cell wall. The cellobiose formed by these reactions was found to be phosphorolytically cleaved, the only member undergoing hydrolysis being cellotriose.

These observations, together with the knowledge that many Cx cellulases are capable of liberating glucose directly have thus indicated that β-glucosidase need not be an essential component of cellulase enzyme systems as implied by Cowling (1963) although

its action may usefully assist in the production of glucose in the case of those organisms possessing Cx components with low affinities for the smaller oligosaccharides and for those organisms only able to utilize the monosaccharide.

Although most of the information available on Cx systems has been obtained as a result of studies performed on modified cellulosic substrates, a significant observation was made by a group of workers who had been concentrating their efforts on the C1 component. Marsh, Merola & Simpson (1953), studying the early changes occurring during in vivo attack of cotton demonstrated the presence of a factor which lead to an increase in the uptake of alkali when the cotton was subsequently placed in 18% sodium hydroxide solution. The same factor was found to be present in culture filtrates prepared from such cellulolytic micro-organisms and was thought to be an enzyme because of its thermolability and the fact that its production could be induced by the presence of suitable substrates. Marsh et al (1953b) subsequently termed this enzyme "S-factor". Further studies by Reese and Gilligan (1954) in an attempt to fit S-factor into the general action pattern of cellulases confirmed that its behaviour was that of an enzyme, and although its identification with Cx activity was not completely clear these workers concluded that S-factor could be regarded as a type of Cx enzyme.

In view of the fact that the probable action of S-factor is to modify some architectural feature present in native cellulose, its behaviour in this respect might be regarded as being analogous to the C1 component. In order therefore to review the theories concerning the possible modes of action of both of these enzymes it is necessary first to consider current concepts of the fine

structure of native cellulose. This review will be confined mainly to the structure found in cotton fibres which are the purest form of naturally occurring cellulose and consequently the most popular choice of unmodified substrate by the majority of workers in this field.

Cotton is the seed hair from plants of the genus Gossypium, each fibre consisting usually of a single cell which originated in the epidermis of the seed coat at about the time of flowering. At this time a cell destined to become a cotton fibre may be distinguished from other types of epidermal cells by an outward extension of the external cell wall. The nucleus migrates towards the growing end of the cell which rapidly elongates to form the fibre. During maturation of the fibre the nucleus may return to a central position, eventually losing its activity and disappearing, (Balls, 1915). During the lengthening period the fibres are essentially circular and when fully elongated exhibit a slight tapering towards the base with a more pronounced tapering towards the tip. Maturation of a fibre consists initially of deposition of cellulose on the internal surface of the primary cell wall, this secondary wall continuing to thicken until a day or two before the cotton boll begins to crack open. The secondary wall never completely occludes the space remaining in the centre of the fibre known as the lumen. Before the boll opens the lumen contains the protoplasmic contents of the cell; opening of the boll by growth processes however causes dehydration and consequent collapse of the cells. The protoplasm dries to a solid residue in the lumen and the fibre falls into a flattened ribbon-like structure with spiral twists. Only at the fibre tip does secondary wall cellulose fill the cross section.

Because of the universal occurrence in wood fibres of heavy deposits of non-cellulosic materials such as lignin and hemicelluloses it has been difficult to study fine details in the walls of fibres from wood. Purification processes necessary to render cellulosic structures visible by microscopy involve such drastic treatments as to throw suspicion on observations made on the purified materials. Fortunately cotton fibres appear to have a structure very similar to that of wood fibres with the exception that the characteristic pits present in the latter are absent from cotton. In cross section both types of fibre consist of six well defined parts; the cuticle, the primary wall, the S1, S2 and S3 components of the secondary wall and the lumen.

The presence of a separate cuticle covering the external surface of the primary cell wall has been difficult to establish conclusively (Rollins & Tripp, 1954; Roelofsen, 1959) due to the fact that on fibre dehydration the cuticle dries on to the primary wall so intimately that it cannot be easily separated. Roelofsen (1959) however regarded the cuticle as that layer remaining after treatment of the fibre with cellulose solvents, implying a non-cellulosic structure. Directly beneath the cuticle lies the primary wall, a structure usually somewhat less than 0.5μ in thickness which can be distinguished from the secondary wall by microscopical observation under polarized light. Under these conditions the primary wall exhibits only slight birefringence in contrast to the highly birefringent secondary wall, indicating that the cellulose in the former is less well orientated towards the axis of the fibre than that in the latter. Roelofsen (1951, 1959) has however expressed doubts as to the validity of most observations made under polarized light due to the frequent

occurrence of furrows in the fibres caused by shrinkage during dehydration. Electron microscopy studies of the primary wall have demonstrated the existence of a loose fibrillar network which has been shown to consist of at least two sets of fibrils; a coarse set running axially on the outer face of the primary wall and a finer set running transverse to the fibre axis on the inner face of the wall (Muhlethaler, 1949; Rollins, 1968). This structure appears to be common in the primary walls of rapidly growing plant cells. The exact dimensions of the individual microfibrils are indeterminate but may be several microns in length with an average width between 100-300 Å. Although the primary wall is composed mainly of cellulose, small quantities of pectin (Rollins, 1968) and waxes (Gundermann et al, 1937) may be present as impurities within the structure.

The bulk of the cellulose content of a cotton fibre occurs in the secondary wall which as described earlier is initiated after the fibre is fully elongated and is now generally accepted as consisting of three distinct layers (Herzog, 1955; Roelofsen, 1959). The texture of the first layer, termed S1, which lies immediately beneath the primary wall appears to be different from that of the bulk of the cellulose constituting the secondary wall by being coarser, with the cellulose fibrils arranged in a banded structure of alternate open and close packing. The bands appear to spiral about the fibre axis at an angle of about 45°. Beneath the S1 layer in mature fibres lies a much thicker layer termed S2 which comprises the major portion of the cell wall. The cellulose in this structure is again laid down in the form of microfibrils which are however finer than those in the S1 layer, and spiral about the axis of the fibre at a slightly less steep angle.

Although the third (S3) layer is not prominent in cotton, Roelofsen (1959) believed such a layer to be present, usually as a thin spiralling structure similar in arrangement and thickness to the S1 layer. In wood fibres however, the S3 layer is often extremely prominent.

The lumen varies in size and shape from fibre to fibre and at different positions along the same fibre. The contents of the lumen are usually regarded as waxes, pectic substances and nitrogenous deposits believed to be the remains of the protoplasm. This material seldom extends the full length of the mature fibre, but is plentiful and often continuous in the lumen of immature cotton fibres.

In cell wall literature the terms "fibril" and "microfibril" have been used more or less indiscriminately to describe the thread-like cellulose filaments observed with the electron microscope. Such observations led to the discussion as to whether the coarser fibrils were bundles of finer ones, and whether or not the finer ones were the ultimate elements from which the larger fibrils were aggregated. Advocates of this theory (Wuhrmann *et al*, 1946; Kinsinger & Hock, 1948) believed that the microfibrils were made up of finer strands termed basic or elementary fibrils, the sizes of which were believed to vary according to the source of the cellulose. In a review by Warwicker *et al*, (1966) a range of cotton microfibril thickness measurements from 80-400 Å is quoted, the elementary fibrils varying between 30 and 160 Å in width. Frey-Wyssling *et al* (1964) however postulated elementary fibrils approximately 35 Å thick to be the ultimate fibrillar unit from which the larger and more frequently measured sizes were aggregated. This theory was supported by Colvin (1964) and Manley (1964),

despite the fact that the majority of microfibrils measured have been found not to be multiples of 35 Å (Gunther, 1960). X-ray evidence does however support the 35 Å elementary fibril concept in the case of the crystalline portions of the microfibrils (Muhlethaler, 1963).

The structure of the elementary fibril is still under considerable discussion, however it is now generally accepted that whatever its dimensions the structure consists of regions of well ordered (crystalline) and less well ordered (amorphous) aggregates of cellulose molecules. From the study of chemical substitution reactions it has been established that cellulose consists of chains of anhydroglucose residues arranged in cellobiose units in a β-configuration, with the C1 atom of the first anhydroglucose residue in ether linkage with the C4 atom of the second. The cellobiose units are linked together in a similar manner to form long continuous chains of cellulose. The spatial arrangement of the hydroxyl groups in these units permits the chains to be linked together laterally by hydrogen bonding, these lateral bonds occurring wherever two or more chains lie in close proximity. Cellulose chains generally lie together in varying degrees of parallelism. When the chains are truly parallel and are close enough for hydrogen bonding to occur, groups of tightly compacted molecules are formed which behave as minute crystals and are termed "crystallites" or "micelles". When the chains do not run truly parallel and are consequently separated to such an extent that few hydrogen bonds are formed, the molecular structure is said to be paracrystalline or amorphous. Alternatively, crystalline regions may be termed "laterally well ordered" and amorphous regions "less well ordered". If of sufficient length, a single cellulose chain

may participate in the formation of several micelles and may terminate within a micelle.

One of the earliest theories on the fine structure of cotton (Nageli, 1858) proposed that micelles were built into the structure like bricks in a wall, but it soon became clear that this did not adequately explain many of the properties of the fibres. Accumulation of evidence eventually led to the formulation of the "fringe-micelle" theory (Meyer & Mark, 1928) which proposed that the crystalline micelles were held together by the fringes of amorphous cellulose chains which formed disordered tangles around each micelle. Hearle (1963) modified this to a fringed-fibril structure in which the crystalline regions were regarded as continuous with amorphous fringes occurring randomly along the length of the fibril. In contrast to these micellar theories, Frey-Wyssling (1959) postulated an idealized structure for the microfibrils which he believed to be formed from four crystalline basic fibrils surrounded by paracrystalline cellulose. Frey-Wyssling however believed the latter to exist in a much higher state of order than the amorphous cellulose envisaged in micellar systems, being composed of highly parallel cellulose chains in almost the correct positions for crystalline cellulose. A further feature repeatedly emphasized by this worker is the existence within the fibrils of a system of voids observed to be of two types; a coarse system into which large molecules can penetrate, and a fine system only accessible after swelling. There is little doubt that this void system in cotton is an important feature of the structure. Many of the reactions attributed to the amorphous regions in the fringe-micelle theory may be equally well explained in terms of long narrow voids of varying width bounded by reactive

cellulosic surfaces. Any changes in the void structure may consequently have profound effects on reactivity.

In an attempt to explain the observation by Ekenstam (1936) that acid hydrolysis of cotton takes place in two stages (a rapid stage followed by a slow stage), various theories concerning the existence of weak links within the anhydroglucose chains have been proposed. For many years it had been accepted that cellulose in cotton was exclusively composed of glucose units. Schulz et al (1942) however postulated that xylose residues occurred regularly every 500 glucose units along the cellulose chain. Pascu & Hiller (1946) however were of the opinion that such acid labile links were of the hemi-acetal type and occurred every 128 glucose residues (approximately every 600 Å). This theory is now almost completely discounted in favour of the acid labile non-glucosidic units. If such units exist in the cellulose chain, their presence should be exhibited by careful chromatographic analysis of hydrolysates. Das et al (1954) claimed to have detected both xylose and arabinose in such hydrolysates, but the evidence available at present does not seem to be extensive enough to prove or disprove the existence of such weak links. Goldstein et al (1957) have claimed that one glucose unit in 500 to 1000 is not linked at the 1:4 positions, but it is not certain whether this irregularity is acid labile. Frey-Wyssling (1959) has attempted to explain Ekenstam's (1936) observation by postulating the existence of small (less than 50 Å in length) amorphous regions disposed regularly along the length of his proposed crystalline and paracrystalline microfibrils. It should be noted however that acid labile links may occur within both crystalline and amorphous regions, and may consequently not be equally accessible to attack.

The quoted lengths for acid hydrolysis fragments may thus be independent of the actual length of chain between the weak links. Whatever the causes of these weak zones, it is now well accepted that they play an important role in mechanical and other properties of cotton. Although they may be zones at which attack can be initiated, they apparently need not be regions accessible to large molecules. If this is so, the principle reactions of cotton must therefore be controlled by the surface area of the fibrils, and hence as mentioned earlier, the complex system of voids within the cotton fibre becomes of paramount importance in determining the subsequent susceptibility to enzymic attack. Stone et al, (1969) investigating the digestibility of wood pulps have in fact concluded that the degree of crystallinity of their substrates had little bearing on the susceptibility to attack, but that the porosity of the sample was the controlling factor in this respect. As mentioned earlier, all cellulosic materials contain in addition to microscopically visible capillaries an appreciable molecular surface resulting from submicroscopic pores. Cowling (1963) has estimated the surface area of the latter in 1g of wood or compacted cotton fibres to be of the order of 3×10^6 cm^2, compared with an area of only 2×10^3 cm^2 bounding the gross void structures. The enormous reactive surface present in the submicroscopic pores is however believed to be physically inaccessible to cellulase molecules (Cowling & Brown, 1969) which are capable of diffusion only into the larger microscopic voids in natural fibres. The significant increase in reactivity of a sample of native cotton following chemical swelling treatments would thus be due to an increase in the dimensions of these submicroscopic pores, allowing ingress of the enzyme molecules.

It would seem clear therefore that digestibility is not solely a function of crystallinity but is also highly dependent upon the porosity of the fibre, a theory in agreement with the observations of Lee (1966) who found that the most reactive of his regenerated celluloses exhibited the highest degree of crystallinity but was also the most porous.

In mature but undried cotton the position is different. The evidence of Berkley & Kerr (1946) that such cotton does not contain crystalline cellulose would exclude the concept of fibrils, despite the belief that elementary fibrils about 35 Å thick are fundamental growth units in native celluloses (Frey-Wyssling *et al*, 1964). Crystalline cellulose was however detected by Sisson (1938) in the early stages of secondary thickening, and Hess *et al* (1939) expressed the view that crystalline cellulose is formed *in vivo* and does not necessarily arise from amorphous regions on fibre dehydration. Early X-ray studies on undried cotton fibres (Berkley & Kerr, 1946) indicated an essentially amorphous structure; observations criticized by Preston (1952) who pointed out that such crystalline regions could be too small to be detected by this method, and that water halos in the diffraction patterns could mask cellulose reflections. More recently Heyn (1965) has concluded that undried cotton does contain some crystalline material though much less than in dried fibres. Whatever the structure of this substrate, there is no doubt that undried cotton fibres removed from mature unopened bolls are much more susceptible to enzymic attack than the fibres which have previously undergone a period of natural dehydration. Although not as reactive as the commonly used grades of soluble cellulose derivatives, undried cotton has been found to be rapidly hydrolysed by many micro-organisms

incapable of catabolizing dried fibres, and by cell-free enzyme preparations inactive against the latter (Marsh et al, 1955; Halliwell, 1964), indicating that the initial action of C1 is not required as a preliminary stage in the hydrolysis of undried cotton fibres by Cx enzymes.

The general consensus of opinion therefore is that the cellulose structure in cotton consists of fibrils which are mainly composed of crystalline material. Disordered regions are envisaged as being confined to the surface of the fibrils and to short regions occurring within the latter. Interspaced in this structure is a complex system of long narrow voids which may be altered by pre-treatment of the cotton, and superimposed upon the whole structure is a system of transverse weak places. This unique structure must therefore be the basis for any theory concerning the possible action of C1 and S-factor enzymes on native cotton fibres.

The change in alkali swelling of a cotton fibre following the action of S-factor is detectable before any reducing sugar, loss in tensile strength or change in DP can be observed (Marsh et al, 1953a; 1953b). Because this change is associated with an increase in fibre width, Reese (1956) suggested that the action of S-factor is chiefly to decrease the dimensionally restrictive effect of the network of fibrils constituting the primary wall of the fibre. In proposing this theory, Reese presumed that most of the tensile strength of the fibre must be derived from the much thicker secondary wall, and believed it possible that S-factor can act merely by breaking a few β 1-4 linkages in suitably orientated primary wall fibrils to relax the constricting effect of the outer layers without producing a simultaneous loss of tensile strength or weight. Alternatively the site of action

may be some non-typical linkage or component. As a result of electron microscopy studies, Rollins & Tripp (1954) suggested that the attack is not restricted to the surface but may also occur deeper within the structure.

For many years following the proposed existence of a C1 enzyme (Reese et al, 1950), all attempts to prepare cell-free extracts capable of producing extensive hydrolysis of native cellulose failed. Some limited success was however achieved by those workers who studied physical changes occurring in the cellulose structure following treatment with enzyme extracts. Blum & Stahl (1952) for example treated dewaxed cotton fibres with an unspecified cellulase extract at 40^{o} and pH 6.0, and although unable to detect any solubilization, change in DP or crystallinity, measured a decrease in tensile strength of the fibres of 34% in 3-5 days. The progress of enzymic attack followed microscopically indicated that the characteristic spiral windings of the fibre disappeared and were replaced by cracks and spiral fissures running in the opposite direction. While severe transverse cracking was observed in the case of mature dried fibres, fibres which had never been allowed to dehydrate exhibited helical splits after enzymic attack.

In 1963, Selby, Maitland and Thompson found that culture filtrates prepared from Myrothecium verrucaria were capable of extensive attack on cotton provided that repeated applications of fresh filtrate were made. Shortly afterwards Mandels & Reese (1964) were successful in preparing even more active extracts from Trichoderma viride which were capable of producing high solubilizations of cotton by a single application, and it became apparent that this would be a more convenient organism to use

in the search for C1. It was also fortunate that at about this time considerable advances were being made in macromolecular fractionation techniques, notably in materials available for gel filtration and ion-exchange chromatography. The following years therefore saw the results of numerous fractionation studies of the cellulase system of T.viride (Mandels & Reese, 1964; Iwasaki et al, 1964; Flora, 1965; Li, Flora & King, 1965; Selby & Maitland, 1967) with the consequent discovery that ten years previously Gilligan & Reese (1954) had in fact come close to isolating the C1 component of this organism by fractionation on calcium phosphate gels. These workers had obtained two fractions between which a synergistic action could be demonstrated, but the involvement of C1 in this action went unobserved at that time because the fractions were only tested against modified cellulosic substrates. The fact that a marked synergism did occur between the C1 and Cx components was demonstrated by Mandels & Reese (1964) and subsequently confirmed by Selby & Maitland (1967), who showed the remarkable effect of this synergism by the application of rigorous fractionation techniques. Thus the C1 of T.viride totally lost the ability to solubilize native cellulose when isolated from Cx. This ability was however completely restored when the isolated components were recombined in their original proportions. In an attempt to explain this loss of activity on purification, Selby & Maitland (1967) suggested that because continued dialysis enhanced the solubilization of cotton by C1 but not by Cx, the C1 enzyme might be strongly inhibited by a soluble dialysable product which Cx was able to remove. The identity or existence of such a product has however remained unresolved.

In addition to T.viride, fractionation studies were also

currently being carried out on extracts from a variety of other cellulolytic micro-organisms including Polyporus versicolor (Petersson et al, 1963); M.verrucaria (Selby & Maitland, 1965); Chrysosporum lignorum (Eriksson & Rzedowski, 1969); Trichoderma koningii (Wood, 1968) and Fusarium solani (Wood, 1969). The most significant of these studies were those published by Wood on T.koningii and F.solani due to the striking similarities exhibited by the cellulase systems of these organisms and that of T.viride. Although Wood was unable to completely free the C1 components of these organisms from residual Cx activity, the ability of C1 to solubilize native cellulose was again lost on purification and restored by recombining the isolated components in their original proportions. Unlike T.viride however the recoveries were not 100% but 77% and 81% respectively. The Cx systems of these three organisms were also found to be very similar, consisting essentially of a CMC-ase which was also capable of hydrolysing insoluble swollen cellulose and was responsible for most of the S-factor activity, and a β-glucosidase component which was also a feeble exo-β-glucanase. Wood (1968) consequently suggested that these similarities indicated one fundamental mechanism for the degradation of native cellulose rather than a random mixture of enzymes, a suggestion subsequently reinforced by this worker's demonstration (Wood, 1969) that the C1 component of F.solani synergized with the Cx components of T.koningii and conversely.

Fractionation studies on M.verrucaria (Selby & Maitland, 1965) however revealed a rather different pattern. Although the system was again found to be heterogenous, consisting of a CMC-ase and two other components responsible for the filtrate's activity towards cotton, Selby & Maitland were unable to demonstrate any

synergism between these three components.

Additional information on the mechanism of degradation of cotton was provided by Halliwell (1965) who showed that cell-free extracts of Cellvibrio gilvus were capable of severing the long fibres of mature cotton to short fibres upto about 3mm in length, this action being accompanied by only a slight liberation of soluble material. Halliwell subsequently investigated a number of other cellulolytic micro-organisms and found extracts of T.koningii to be even more active in this respect, with the early enzymic breakdown of the cotton fibres being again characterized by the formation of very short fibres (Halliwell & Riaz, 1970). These fibres increased to a maximum level with only slight simultaneous production of soluble materials, and were then gradually hydrolysed to glucose. Short fibre production was considerably assisted by shaking, with approximately 80% of the cotton existing as short fibres and 16% as soluble sugars after 20 hours incubation at 37°. Halliwell did not however make any attempt to identify C1 action with the observation of short fibre liberation, although the presence of a Cx component active on both CMC and reprecipitated cellulose, and a β-glucosidase component was established. More recently Halliwell & Riaz (1971) resolved the cellulase complex of T.koningii into four components; C1, β-glucosidase, and two components which Halliwell and Riaz termed CMC-ase and C2. It was claimed that all four components were necessary for the efficient solubilization of native cellulose, with CMC-ase and C2 together being responsible for short fibre production (Halliwell & Riaz, 1970). It is interesting to note that the fractionation studies on T.koningii carried out by Wood (1968) did reveal the existence of a fourth component found to be

a low molecular weight CMC-ase which Wood however found had no effect on the ability of the enzyme complex to solubilize cotton. The existence of a similar component in T.viride extracts had also been reported earlier by Selby & Maitland (1967). Although the results obtained by Halliwell & Riaz (1971) indicate that C1 is not involved in short fibre production in the case of T.koningii, the action of this enzyme in a similar fragmentation phenomenom was demonstrated in the case of T.viride by King (1966) and Liu & King (1967). These workers observed that a partially purified C1 extract produced a marked increase in the total number of particles in a hydrocellulose substrate; ie a particulate highly crystalline residue formed by carefully controlled acid hydrolysis of native cellulose. The small particles liberated were believed to be micelles which were known to be bound together by hydrogen bonds in hydrocellulose preparations. These observations prompted Liu & King to suggest that the C1 component might not be an enzyme in the usual sense, but rather a protein which hydrogen bonded to cellulose more tightly than the cellulose hydrogen bonds themselves. This it was postulated would result in the gradual collapse of the particles to release the micelles, thereby increasing the surface area available for further attack. Selby (1969) however expressed the belief that similar disaggregation could not be presumed to occur in cotton fibres in which the more structurally complex fibrillar bundles would be less likely to separate in this manner, despite the fact that particles about the size of individual micelles may be formed when cotton is treated with whole culture filtrate.

The role of the C1 component in the cellulase complex has therefore remained unresolved more than twenty years after its

existence was first postulated. Opinions have been expressed from time to time concerning the possible identity of C1 with S-factor in view of the fact that both components are presumed to be involved in the early stages of attack on native cellulose. In addition, swelling may reasonably be considered to be a necessary prerequisite of hydrolysis. The original suggestion by Reese and Gilligan (1954) that S-factor is a property of the Cx system has however been subsequently confirmed by several workers (Youatt, 1962; Nisizawa et al, 1966; Wood, 1968; 1969). The importance of the postulated role of S-factor in increasing the susceptible surface area of the substrate has also recently been questioned with the observation that the rate of adsorption of T.viride C1 by the substrate was unaffected by the presence of Cx, and that of Cx by C1 (Selby & Maitland, 1967). These workers failed to detect any function which C1 alone could perform that was not equally a property of Cx or of a mixture of these enzymes, and consequently concluded that both C1 and Cx must be present on the substrate simultaneously when contributing to the solubilization of highly ordered cellulose.

The inactivity of purified C1 towards native cellulose measured by the production of short polyanhydroglucose chains as originally postulated by Reese et al (1950) has led to a critical re-examination of this theory. Shortly after the existence of C1 was proposed, Siu (1951) light-heartedly suggested that rupture of hydrogen bonds might form an essential part of this enzyme's action, a proposal which became more feasible with the subsequent observation of hydrocellulose fragmentation by C1 (King, 1966). More recently Selby (1969) has postulated a new theory of C1-Cx action based on this "hydrogen-bondase" concept

and on new theories concerning the supramolecular structure of cotton (Warwicker *et al*, 1966) in which the fibre is believed to be formed of completely crystalline elementary microfibrils each containing about 100 cellulose chains. Selby believes that the disturbance in hydrogen bonding occurring in the vicinity of the chain ends may be insufficient to allow Cx alone to split off soluble sugars. When both C1 and Cx are present however, a single bond rupture by Cx might allow the hydrogen bonding to be further disturbed by C1 with a consequent loosening of the structure, permitting more extensive attack by Cx. It will be apparent that this concept departs markedly from the classical theory in that C1 is no longer regarded as performing the initial attack. In addition to being based on the "hydrogen-bondase" theory, Selby's proposals contain the added notion of a very limited number of sensitive sites to explain the difficulty in detecting the action of the purified cellulase components when acting alone on the cotton fibre.

At the time of commencement of this investigation it was thus apparent that our knowledge of the enzymic dissolution of native cellulosic materials was extremely limited. Although the Cx enzymes of a wide variety of micro-organisms had been thoroughly investigated, the C1 component despite its obvious importance in this process had been largely neglected. The few reports available at that time describing this enzyme (Mandels & Reese, 1964; Li, Flora & King, 1965; Flora, 1965; Selby & Maitland, 1967; Wood, 1968) were concerned mainly with its isolation from the rest of the cellulase complex of either *T.viride* or *T.koningii*, and furnished few details of its possible mode of action. As an active programme of research on *Trichoderma* was obviously well

established, a similar investigation carried out on a different organism was considered necessary to test Wood's fundamental concept of a microbial cellulase system, and to study the mechanism of C1 action in some detail. In addition it was hoped that some information could be obtained on the possible occurrence of cell-bound cellulase components in an effort to explain the considerable differences in activity exhibited by cell-free extracts and the viable micro-organism.

The decision to investigate cellulase activity in Cephalosporia was dictated by several factors. As part of a research programme being carried out at the University of Bath on morphological and physiological features of this group of organisms, a preliminary screening of the University culture collection had revealed the presence of a number of actively cellulolytic species (Wright,1968). The ability of these organisms to solubilize highly ordered forms of cellulose was also apparently dependent upon the proximity of the organism to the substrate, suggesting a cell-bound or non-diffusible cellulase component. Evidence indicating the importance of Cephalosporia in the natural decomposition of plant wastes had been provided by Dickinson & Pugh (1965) who by the use of a selective cellulose agar observed these organisms to be the most frequently isolated cellulolytic soil fungi. Domsch and Gams (1969) subsequently examined the ability of several fungi to hydrolyse a variety of polysaccharides including CMC, and reported Cephalosporium roseum and C.furcatum to be even more active on this substrate than many of the more well known cellulolytic fungi including Trichoderma, Myrothecium, Fusarium, and Penicillium.

A detailed analysis of the cellulase system of this important

but apparently largely ignored group of organisms was consequently considered to be a potentially worthwhile contribution to the field of cellulase research.

MATERIALS AND METHODS

(a) Selection of organism

The organism used throughout this investigation was selected from a culture collection of *Cephalosporia* and related organisms on the basis of a preliminary screening for cellulolytic activity carried out as part of a final year degree project (Wright, 1968). This screening was based on measurement of solubilization zones produced in cellulose agar plates by each organism. Initially, standard plastic petri dishes (Sterilin) were poured with 15.0 ml aliquots of the medium described by Skinner (1960) containing

0.05%	w/v	$(NH_4)_2SO_4$
0.65%	"	K_2HPO_4
0.35%	"	KH_2PO_4
0.005%	"	$CaCl_2$
0.005%	"	$MgSO_4.7H_2O$
0.1%	"	NaCl
0.01%	"	Yeast extract (Oxoid)
0.5%	"	CMC (sodium salt)
0.05%	"	Cellulose
1.5%	"	Agar (Oxoid No.3)

The cellulase substrate was added in the form of an insoluble particulate suspension in distilled water, produced by treating non-absorbent cotton wool with 90% v/v hydrochloric acid for 24h at room temperature, washing with distilled water until neutral and ball milling for 72h in a Pascall mill, (Hungate,1950). The medium was sterilized by autoclaving at 15 lb pressure (121°) for 15 min and allowed to cool to 45° before pouring. Despite this cooling and incorporation of CMC into the medium, considerable

sedimentation of the cellulose particles occurred before the agar solidified. Subsequent growth of the test organism was sparse, and no areas of cellulose hydrolysis could be observed after incubation at 27° for 21 days. In order to overcome this problem a layer plate technique was adopted. Plates were poured initially with 10.0 ml aliquots of the medium described previously but lacking the cellulose substrate, and allowed to solidify. 5.0 ml of the complete medium at 45° were spread evenly over the first layer and gelling was accelerated by immediately transferring the plates to a deep freeze compartment at -20° for 10 min. Microscopical examination of sections cut from these plates revealed that the cellulose particles were evenly distributed throughout the upper layer, the lower layer acting as a nutrient reservoir and preventing premature desiccation of the medium.

Plates were stab inoculated in triplicate from spore suspensions of each organism prepared by shaking 2.0 ml of sterile distilled water and a few sterile glass beads with cultures grown on malt agar (Oxoid) slopes for 14 days at 27°. After incubation at 27° for 7 days the plates were examined for growth, and colony and cellulose solubilization zone diameters measured using vernier calipers. For further comparison the extent of cellulose hydrolysis was assessed in each case on an arbitrary scale from 0 (no visible clearing) to 5 (extensive clearing). After this incubation period cellulose decomposition became obscured by hyphal growth. The results of this screening are reproduced in Table.1.

(b) Maintenance of cultures.

Stock cultures were maintained in sterile soil at 4°, subculturing being performed by aseptically transferring a few soil crumbs to

fresh medium as required. At regular intervals subcultures were made on malt agar plates incubated at 27° for 14 days and on nutrient agar plates incubated at 27° and 37° for 7 days as a check for fungal and bacterial contamination.

(c) Substrates.

Glucose, cellobiose, o-nitrophenyl β-D glucopyranoside and 6-bromo-2-naphthyl β-D glucopyranoside were purchased from BDH Ltd., Poole, England. Cellotriose and cellotetraose were prepared by acid hydrolysis of Whatmans CF-11 cellulose powder with fuming hydrochloric acid. After neutralization of the hydrolysate with sodium bicarbonate the oligosaccharides were isolated by chromatography on a charcoal-celite column prepared with BDH activated charcoal and eluted with an increasing ethanol gradient (Miller, Dean & Blum, 1960).

Sodium carboxymethyl cellulose was a sample obtained from British Celanese Ltd. with an average degree of substitution of 0.5 as measured by the method of Green (1963).

An amorphous particulate cellulose substrate was prepared by the method of Hungate as described under (a).

Native cotton fibres were obtained from the fully opened bolls of plants of Gossypium barbadense kindly provided by the Horticulture department of the University of Bath. Samples of undried boll cotton were obtained by dissecting ripe but unopened bolls under water, storing the fibres until required in 0.005 M sodium azide at 5°. Non-absorbent cotton wool was also utilized as an alternative source of highly ordered crystalline cellulose.

(d) Enzyme production in liquid culture.

Organisms were cultured in 250 ml Erlenmeyer flasks containing 80.0 ml of medium and incubated at 27° on a rotary shaker (320 rpm,

1" throw). The liquid medium used throughout this investigation for the growth of C.acremonium consisted of the mineral salts-yeast extract base described earlier to which was added various cellulose derived carbon sources at a level of 1.0% (w/v). Sterilization was by autoclaving at 15 psi for 15 min. In the case of glucose and cellobiose precipitation of phosphates was found to occur; this was prevented by autoclaving 40.0 ml of double strength mineral salts solution and 40.0 ml of 2.0% glucose or cellobiose in distilled water in separate flasks and mixing aseptically when cool.

Seed stage flasks containing the mineral salts/glucose medium were inoculated from the stock soil cultures by the method described earlier and shaken at 27° for 5 days. Cells were harvested by centrifugation at 23,000g in an MSE 18 high speed centrifuge at 0°, washed twice with chilled sterile distilled water and finally resuspended in the latter to give a suspension which when diluted one in ten showed 50% transmission at 600 nm in an EEL Spectra colorimeter. 2.0 ml aliquots of this suspension were used as inoculum for the production stage flasks containing oligosaccharides or cellulosic substrates.

Optimum incubation periods for enzyme induction were ascertained by assaying culture filtrates prepared from production stage flasks at 24h intervals. These standard inoculation and incubation procedures were maintained throughout subsequent investigations.

(e) Enzyme extraction.

In addition to the cell-free components of the cellulase system present in culture filtrates, the possible existence of cell-bound components was also investigated.

Cultures were centrifuged at 23,000g for 30 min at 0°, the

supernatant decanted into medical flat bottles and frozen at -20° as a thin layer on the broad side of the bottle before being transferred to the vacuum chamber of a freeze drier. The cells were washed once with citrate buffer pH 5.0 (0.05 M) and recentrifuged for 10 min. Approximately 10.0g of wet cells were suspended in 30.0 ml of citrate buffer and homogenised with 50.0g of Glasperlen beads (0.45-0.50 mm diameter) for 5 min in a Braun (M.SK) homogeniser at maximum speed. The homogenate was centrifuged at 38,000g for 30 min at 0° and the supernatant removed. The cell fragments were washed once with citrate buffer, recentrifuged at 38,000g for 15 min, frozen at -20° and freeze dried.

Freeze dried filtrates and cell fragments were stored at 5° for a maximum period of four weeks and were then discarded due to gradual loss of activity. The cell-free enzymes were obtained as required by reconstituting the freeze dried filtrates with 0.05 M citrate buffer pH 5.0 at 5°. In the early stages of this investigation the cell bound cellulase activity was estimated in a crude enzyme preparation consisting of a 1.0% w/v suspension of cell fragments in citrate buffer. In order however to compare the properties of the cell-bound components with those found free in culture filtrates it was found necessary to obtain samples of the bound enzymes in aqueous solution. Several solubilization treatments were tested by suspending 0.1g amounts of cell fragments in 10.0 ml quantities of (i) 0.2 M NaCl; (ii) 0.2 M $CaCl_2$; (iii) 8.0 M urea; (iv) distilled water; (v) 0.05 M citrate buffer pH 5.0 at room temperature for 2h. In addition, an enzyme preparation obtained from a soil streptomycete capable of degrading cell walls of C.diospori but shown in controlled experiments to be free from any cellulolytic activity was investigated for its

efficiency in releasing cell-bound cellulase components by suspending 0.1g of cell fragments in 6.0 ml of this enzyme solution and 4.0 ml of citrate buffer pH 5.0 at 30° for 2h. After treatment the cell debris was removed by centrifugation at 4,000 rpm in a BTL bench centrifuge for 5 min, the supernatants collected and the debris resuspended in 10.0 ml quantities of fresh citrate buffer. Samples of the supernatants and the treated cell fragment suspensions were withdrawn and tested for cellulolytic activity, and these results were then compared with the activity exhibited by a 1.0% w/v suspension of untreated fragments in citrate buffer.

(f) Enzyme assays.

(i) Cx enzymes. Cellobiase (β-glucosidase) activity was determined by incubating 1.0 ml of an appropriate dilution of enzyme solution in citrate buffer pH 5.0 with 1.0 ml of a 5.0 mM solution of o-nitrophenyl β-D glucopyranoside (ONPG) in citrate buffer containing 0.005 M NaN_3 for 20 min at 40°. The reaction was terminated by the addition of 2.0 ml of molar Na_2CO_3 solution, the volume adjusted to 10.0 ml with distilled water and the optical density of the solution at 420 nm read against a suitable blank. Optical density readings were converted to μg of o-nitrophenol liberated by reference to a standard curve. One unit of β-glucosidase activity was defined as the amount of enzyme required to liberate 1.0 μg of o-nitrophenol under the conditions of the assay. Reaction progress curves were produced using the assay method described above to analyse samples withdrawn at suitable intervals from incubation mixtures containing 8.0 ml of ONPG solution made 0.005 M with respect to NaN_3, 1.0 ml citrate buffer pH 5.0 and 1.0 ml of enzyme solution. Controls consisted of enzyme and

substrate solutions diluted similarly with buffer and incubated separately.

Activity towards oligosaccharides was measured in digest mixtures consisting of 1.0 ml of enzyme solution and 1.0 ml of a 5.0 mM solution of these substrates in citrate buffer containing 0.005 M NaN_3. The mixture was incubated at 40° for 20 min and the increase in reducing power estimated by adding 2.0 ml of Somogyi (1952) reagent, heating at 100° for 15 min and cooling. 2.0 ml of Nelson (1944) reagent were added, the mixture diluted to 25.0 ml and the optical density at 660 nM read against enzyme and substrate blanks incubated separately under the same conditions. Optical density readings were converted to µg of reducing sugar (as glucose) by reference to a standard curve. Under these conditions the amount of hydrolysis was proportional to the enzyme concentration, and the unit of activity was defined as the amount of enzyme required to liberate reducing sugars equivalent to 1.0 µg of glucose under the conditions of the assay.

Enzymatic hydrolysis of carboxymethyl cellulose was estimated either viscometrically or by measuring the production of reducing sugars. Viscometry studies were carried out in an Ostwald viscometer (Technico VS 220B) mounted vertically in a thermostatically controlled glass water bath at 40°. A 1.0% w/v solution of CMC was prepared by heating the substrate in citrate buffer at 100° for 30 min followed by centrifugation at 4,000 rpm for 15 min to remove particulate matter. The clear supernatant was made 0.005 molar with respect to NaN_3 and 8.0 ml added to the viscometer to equilibrate. 1.0 ml of citrate buffer was added followed by 1.0 ml of enzyme solution; both of these solutions were raised to 40° prior to addition by incubation separately in the same water bath

for a few minutes. The time was noted when 0.5 ml of the enzyme had entered the viscometer, and the contents mixed for 1 min by drawing the mixture repeatedly through the capillary of the viscometer. Measurements of the time required for the liquid meniscus to pass two graduation marks were made at regular intervals after the addition of the enzyme. The experiment was repeated using 1.0 ml of the same enzyme solution denatured by heating at 100° for varying periods, and finally after thorough cleansing, the viscometer was loaded with 10.0 ml of distilled water at 40° and the flow time measured. A graph of relative viscosity (nR) against reaction time was plotted, where

$$nR = \frac{\text{time of flow of incubation mixture}}{\text{time of flow of water}}$$

and $$\text{Reaction time} = x + (y/2)$$

where x is the time between mixing enzyme and substrate and the start of any viscosity measurement, and y is the flow time of the incubation mixture (Wood, 1955).

The action of enzyme inhibitors on the hydrolysis of CMC could be studied by substituting 1.0 ml of inhibitor solution in place of the 1.0 ml aliquot of buffer included in uninhibited mixtures, thereby maintaining the final volume at 10.0 ml. By withdrawing suitably sized samples from identical incubation mixtures maintained at 40° in a separate water bath and assaying for reducing sugars, a direct comparison of the hydrolytic process could be made as measured by both methods. When assaying routinely for CMC-ase activity however, 1.0 ml of enzyme solution was incubated with 1.0 ml of 1.0% CMC solution for 2h at 40° and tested

for reducing sugar content. The unit of activity was defined as that amount of enzyme required to liberate reducing sugars equivalent to 1.0 μg of glucose under the conditions of the assay.

Hydrolysis of the acid swollen milled cellulose substrate was determined routinely by incubating 1.0 ml of a 2.0% w/v suspension of the substrate in citrate buffer with 1.0 ml of enzyme solution for 2h at 40° and testing for reducing sugar production. The unit of activity was the same as that defined for CMC-ase. The progress of enzymic hydrolysis of this substrate was studied by estimating reducing sugar levels in samples withdrawn at suitable intervals from incubation mixtures containing 8.0 ml of the cellulose suspension made 0.005 M with respect to NaN_3, 1.0 ml of citrate buffer pH 5.0 and 1.0 ml of enzyme solution. Residual cellulose was removed by centrifugation at 4,000 rpm before optical densities were read. Controls consisted as previously of enzyme and substrate solutions incubated separately.

(ii) Cellulase The solubilization of native cotton fibres was examined initially by assaying for the liberation of reducing sugars in incubation mixtures containing 100 mg of cotton fibres, 1.0 ml of citrate buffer and 1.0 ml of enzyme solution. As previously, mixtures were protected from contamination by the addition of sodium azide to give a final concentration of 0.005 M. Crude enzyme extracts such as reconstituted freeze dried filtrates were centrifuged at 38,000 g for 30 min at 0° to remove particulate matter before being added to the incubation mixtures which were then incubated at 40° for 7 days in 15.0 ml tapered glass centrifuge tubes. Controls again consisted of substrate and

enzyme preparations incubated separately under the same conditions. At the end of this period the residual cellulose was sedimented by centrifugation at 3,000 rpm and the supernatant carefully removed by means of a Pasteur pipette. Samples obtained in this manner were usually free from visible fragments of residual substrate, but as a safeguard the samples were filtered before assay by means of sintered glass (grade 3) filter sticks. The unit of cellulase activity was defined as that amount of enzyme liberating 1.0 µg of reducing sugar under the conditions of the assay. When however no activity could be measured by this method, portions of the remaining samples were assayed for total soluble carbohydrate content by the phenol-sulphuric acid method described by Dubois et al (1956). As a further check in some cases the residual cellulose was estimated by oxidation with dichromate-sulphuric acid reagent (Selby & Maitland, 1967).

The course of hydrolysis of this and other fibrous non-dispersed substrates could not be followed over a period of time by employing the sampling techniques used previously due to the inhomogeneity of the incubation mixtures. Consequently in such investigations it was necessary to prepare identical sets of incubation mixtures and controls, and to assay a complete set in duplicate at regular intervals of time.

(g) Enzyme purification- (i) Column chromatography. Fractionations of culture filtrates and solubilized cell bound enzymes on Sephadex gels were carried out at 5^{o} in Pharmacia chromatography columns K15/90 and K25/100, the gels being equilibrated and eluted with 0.05 M citrate buffer pH 5.0 containing 0.005 M NaN_3. The columns were fitted with constant head devices and the gel beds packed by applying hydrostatic pressure according

to the manufacturer's recomendations for each grade of gel. After the passage of two column volumes of buffer at this pressure, the void volumes of the beds were determined by eluting a blue dextran marker solution.

Before fractionation, enzyme extracts were loaded with sucrose to a final concentration of 10.0% w/v, and the mixtures centrifuged at 38,000 g for 30 min at 0^{o}. The addition of sucrose increased the density of the extracts sufficiently to allow them to be applied to the gel bed beneath the surface of the eluant by means of a hypodermic syringe fitted with fine bore (0.5 mm ID) flexible polythene tubing (Portland Plastics Ltd.). 1.0 ml samples were applied to the K15/90 column and 3.0 ml fractions collected; 3.0 ml samples were applied to the K25/100 column and 10.0 ml fractions collected using in both cases a syphon operated fraction collector (Central Ignition Co., London). Later in this investigation the K25/100 column was fitted with a flow adaptor and fractionations performed by upward flow elution. This was found to give a finer control of flow rate and allowed enzyme application to be simplified by the use of a three way tap fitted into the inlet of the column. Fractions were assayed for cellulolytic activity as described under (f) and stored at -20^{o} until required.

(ii) Disc electrophoresis.

Disc electrophoresis was carried out according to the method of Ornstein (1964) and Davis (1964) using 7.5% acrylamide small pore gel pH 9.5 polymerized in precision bore (5.0 mm ID) glass tubes 85 mm in length. Satisfactory results were only obtained however when careful attention was paid to preparing extremely clean enzyme extracts. Crude extracts such as culture filtrates were

therefore initially purified and desalted on Sephadex G75 in a Quickfit CR 32/40 column with distilled water at 5° as eluant. The desalted protein eluted in the void volume was estimated by the method of Lowry et al (1951), deep frozen at -20° and freeze dried. The residue was reconstituted with sufficient tris-glycine buffer (0.01 M, pH 8.3) containing 0.25 M sucrose to give a final concentration of 250-300 µg protein in 50-100 µl of solution. This concentrated extract was centrifuged at 38,000 g for 1h at 0°, and 250-300 µg of protein applied to each gel. With 0.01 M tris-glycine buffer pH 8.3 as the reservoir buffer for both upper and lower electrode compartments, a constant direct current of 2.5 mA per tube was applied by means of a VoKam SAE 2761 power pack. 1.0 ml of 0.002% bromophenol blue was added to the upper electrode buffer as a marker dye, and electrophoresis continued until this dye marker band reached the base of the gels. After removal from the tubes the gels were stained to exhibit all protein bands by immersion in 0.3% naphthalene black in 7.0% acetic acid for 1h, followed by destaining to remove unbound dye by repeated washings of 7.0% acetic acid over a period of about 2 days.

Several methods for locating the position of specific cellulolytic enzymes in the gels were attempted. β-glucosidase was initially identified by incubating identical unstained gels in 0.005 M o-nitrophenyl β-D glucopyranoside in citrate buffer pH 5.0 at 40° for about 30 min, followed by immersion in M Na_2CO_3. A very diffuse band of o-nitrophenol was observed which quickly spread through the gel, making comparison with naphthalene black stained gels difficult. Satisfactory results were however obtained when 6-bromo, 2-naphthyl β-D glucopyranoside (BNG) was

substituted as artificial substrate for β-glucosidase. The product of cleavage, 6-bromo 2-naphthol is somewhat insoluble and is strongly adsorbed by proteins capable of liberating this chromogenic product. It is coupled in situ with tetrazotised o-dianisidine to give, according to the BDH information sheet, an intense blue colour at sites of enzyme activity. Using the following method it was in fact found to produce a red-brown band in acrylamide gels. 10.0 mg of BNG were dissolved in 50.0 ml of citrate buffer pH 5.0 by vigorous shaking for several hours. Gels were immersed in 10.0 ml portions of this solution at 40° and about 5 mg of Diazo Blue B salt (K & K Laboratories Ltd, New York) added. After incubation for 15 min the gels were washed with distilled water and observed; diffusion of the bands was found to be very slight even after several months storage.

Attempts to locate the positions of other cellulolytic enzymes were made by preparing 1.0% solutions of CMC or 1.0% suspensions of acid swollen cellulose in citrate buffer pH 5.0 containing 1.5% Oxoid agar No.3. After heating to 100° to dissolve the agar the substrates were cooled to 45° and 15 ml portions added to sterile plastic petri dishes. A freshly run acrylamide gel was carefully placed in the centre of the plate and allowed to become embedded as the agar solidified. The plates were then incubated at 40° for periods of upto 21 days. Hydrolysis of the acid swollen cellulose was examined for by noting any areas of clearing along the length of the gels; zones of CMC hydrolysis were made visible by flooding the plates containing this substrate with 10.0% w/v copper acetate solution, rendering undegraded CMC opalescent.

Preparative disc electrophoresis studies were performed in

a column constructed from clear seamless Perspex tubing, the final dimensions of the column being 120 mm x 13 mm OD x 10 mm ID. Approximately 5mm from one end, two opposite Perspex side arms (20 mm x 5 mm OD x 3 mm ID) were fitted to the column and sealed in position by means of a solution of Perspex dissolved in chloroform. A 50 mm length of 10 mm diameter cellulose acetate dialysis tubing was attached to one side arm and connected to a silicon rubber feed line from a Quickfit 6 channel peristaltic pump. The other side arm was fitted with a No.0 serum hypodermic needle by means of a short silicone rubber connector, and the needle connected to a suitable length of flexible polythene capillary tubing 1.0 mm ID.

The column was prepared by inserting the broad end of a 10 mm rubber bung into the lower end of the column until the side arm openings were just sealed. 5.0 ml of 7.5% small pore acrylamide solution were added and allowed to polymerize by overlayering with distilled water from a fine hypodermic syringe. The water was removed and the surface of the gel dried with a tissue before the addition and polymerization of 1.0 ml of stacking gel solution. The rubber bung was carefully removed with a twisting action so as not to dislodge the running gel from the wall of the column and then replaced to a level just below the side arm openings, forming a small elution chamber. 0.55 M citrate buffer pH 5.0 was pumped through the dialysis tubing and elution chamber until all air bubbles had been flushed from the system, and the column was clamped in a vertical position with its lower end and dialysis tubing immersed in the lower electrode (anode) buffer contained in a small perspex trough. A similar trough was attached to the upper end of the column by

Figure 26.

Preparative Disc Electrophoresis Column.

KEY.

a) Upper electrode chamber.

b) Carbon cathode.

c) 0.1 M Tris-glycine buffer pH 8.3.

d) Rubber bung.

e) Large pore stacking gel.

f) Small pore running gel.

g) Citrate buffer delivery from peristaltic pump.

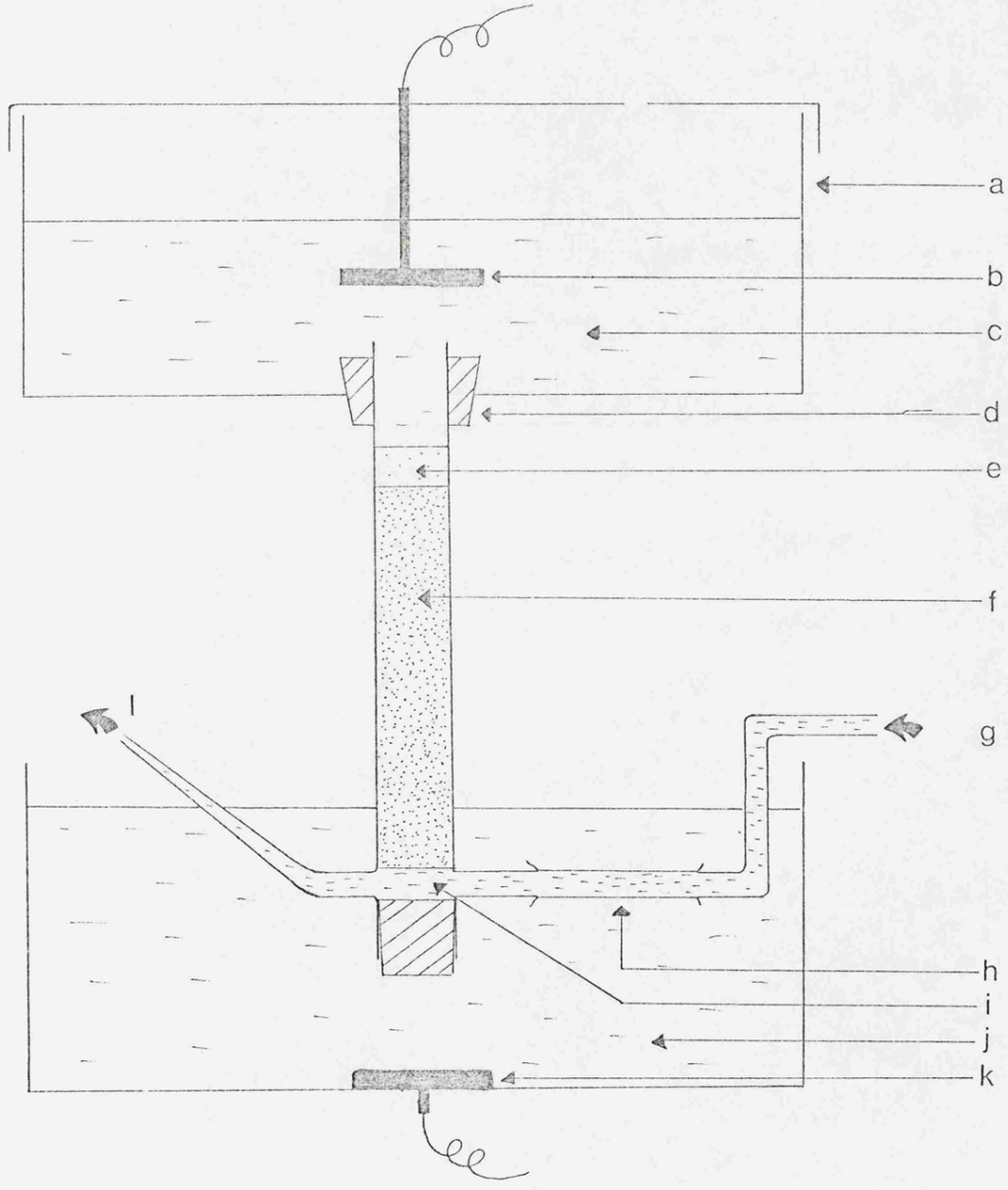
h) Section of cellulose acetate dialysis tubing.

i) Elution chamber.

j) 0.55 M citrate buffer pH 5.0.

k) Carbon anode.

l) Delivery to fraction collector.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means of a rubber grommet, and 0.5 ml of enzyme extract containing up to 4.0 mg protein / ml applied to the gel. The upper electrode reservoir was filled with 0.1 M tris-glycine buffer pH 8.3, and the lower with 0.55 M citrate buffer pH 5.0. Carbon electrodes connected to a VoKam power pack were immersed in the buffers and a constant direct current of 10 mA at 250-300 volts was applied. The elution chamber was flushed at the rate of 8.0 ml/h and 3.0 ml fractions collected by means of a syphon operated fraction collector. The apparatus was prepared and run overnight in a cold room at 5°, (fig 26).

(iii) Ion exchange chromatography.

DEAE-Sephadex (A-25) was equilibrated in 0.1 M acetate buffer pH 8.0 and eluted with a linear decreasing pH gradient with acetate buffer. SE-Sephadex (C-25) was equilibrated in 0.1 M acetate buffer pH 4.0 and eluted with a linear increasing pH gradient with acetate buffer. Fractionations in both cases were carried out at room temperature in Quickfit CR 32/40 columns, collecting 1.5 ml fractions by means of a drop counter operated fraction collector. Because the fractionations were performed at room temperature however, the fractions were immediately removed from the collector and deep frozen until required for assay.

(iv) Selective thermal denaturation.

1.0 ml portions of freeze dried culture filtrates reconstituted in citrate buffer pH 5.0 were heated at 60°, 70°, 80°, 90° and 100° for 1 min in constant temperature water baths before the addition of substrates. The mixtures were then incubated at 40° and assayed by the standard methods described earlier. The experiment was then modified so that enzyme preparations were maintained at 60° for 1, 2, 3, 4 and 5 min intervals before

incubation with the substrates. Controls in both cases were prepared by similarly treating enzyme solutions previously denatured by heating at 100° for 15 min.

(h) pH profiles

The effect of pH on enzyme activity was determined by carrying out standard assays at 40° in McIlvaine (citrate-phosphate) buffer pH 3.0 - 9.0, using samples of isolated enzymes obtained by column chromatography.

(i) Temperature profiles

Enzyme activities at various temperatures were assayed in the standard manner in citrate buffer pH 5.0 by incubating solutions of isolated enzymes and their substrates in thermostatically controlled water baths ranging between 5° and 75°.

(j) S-factor determinations

2.0 ml samples of isolated enzymic components in citrate buffer pH 5.0 were incubated at 40° for 12h with 20 mg of native cotton fibres. At the end of this period the fibres were removed from the incubation mixtures, placed on filter papers to absorb excess moisture and immersed in 18% NaOH for 1h. After centrifugation at 3,500 rpm for 1 min in a perforated centrifuge cup the fibres were reweighed. Controls consisted of fibres incubated in citrate buffer pH 5.0.

(k) Tensile strength determinations

1.0 g samples of unmercerized cotton thread were immersed in 20.0 ml portions of isolated enzymic components and incubated at 40° for 12h. The threads were washed once with distilled water and the wet breaking points determined by means of a Hounsfield tensometer. The average of five determinations for each treatment was noted, controls consisting as previously of threads incubated

in citrate buffer pH 5.0.

(l) Molecular weight estimations

3.0 ml samples of 5.0 mg/ml solutions of catalase (MWt 225,000), bovine serum albumin (70,000), ovalbumin (45,000), pepsin (35,500) and lysozyme (14,700) were applied to the same column of Sephadex G200 used for enzyme fractionations and eluted with citrate buffer, collecting 10.0 ml fractions. The samples were located by measuring the absorbance of the fractions at 280 nm in a Unicam SP 500 spectrophotometer against a citrate buffer blank and their elution volumes (Ve) noted. The void volume of the bed (Vo) was determined by eluting a 3.0 ml sample of 0.1% blue dextran solution, and a graph of Ve/Vo against log MWt plotted (Whitaker, 1963).

(m) Enzyme-substrate affinities

Affinities of isolated components of the cellulase system for various cellulosic substrates were ascertained by carrying out standard assays using substrate solutions or suspensions of varying concentration.

(n) Identification of products of enzyme attack

(i) Paper chromatography.

2.0 ml samples of incubation mixtures were desalted in an electrolytic desalter, freeze dried and reconstituted with 0.2 ml of distilled water to give a ten-fold concentration. 20.0 µl samples were applied to Whatman No.1 chromatography paper and developed in iso-propanol/water (4:1). Sugars were located by dipping the papers in aniline reagent (1.3 ml aniline in 50.0 ml acetone; 0.6 ml phosphoric acid in 20.0 ml acetic acid plus 30.0 ml acetone, solutions prepared separately and mixed as required) and heating at 100° for 5 min (Smith, 1960). Pentoses produce

red-brown spots, other reducing sugars yellow-brown spots on a yellow background; chromatograms dipped in this reagent were found to be quite stable and did not fade.

(ii) Column chromatography

High molecular weight products of enzymic hydrolysis of cellulose and carboxymethyl cellulose were identified on the basis of molecular size by applying 1.0 ml samples of incubation mixtures to Quickfit CR 32/40 columns packed with either Sephadex G15 (fractionation range up to 1,500) or G25 (fractionation range 100-5,000) and eluting with distilled water. 0.5 ml fractions were collected using a drop counter fraction collector and assayed for total carbohydrate by the phenol-sulphuric acid method (Dubois et al, 1956).

(o) X-ray crystallography studies

Debye-Scherrer powder patterns of samples of cellulose were obtained using a Unicam single crystal camera (30 mm radius) with a copper target and nickel filter, giving a Cu K_α line of 1.5418 Å. The samples were packed into 0.5 mm bore low absorption glass Lindemann tubes (Pye Unicam, Cambridge) and rotated at 1 rpm during exposure for 5h.

(p) Refractive index studies of cellulose by microscopy

Comparison of the refractive indices of native cotton and the residual short fibres liberated from this substrate by the action of C.acremonium was made by a modification of the immersion method described by Rollins & Tripp (1963). Samples of each type of fibre were placed about 10 mm apart on clean glass microscope slides and held in place by 30 mm cover slips. A range of liquids with refractive indices varying between 1.540 and 1.570 in increments of approximately 0.002 unit was prepared by mixing varying amounts

of benzyl alcohol and benzyl benzoate, and the fibres immersed in these solutions by applying approximately 0.1 ml of the liquid to the edge of the cover slips. Each slide was then examined by means of a binocular microscope, focusing on the fibres until the bright Becke line (produced at the interface of a solid immersed in a liquid of different refractive index) was visible. The direction of movement of the line when the focus of the microscope was raised was noted in the first 25 fibres observed in each sample, and the refractive index of each solution accurately measured by an Abbe refractometer illuminated with a sodium D line (5896 Å) at 21°.

RESULTS

(a) Selection of organism

On the basis of the preliminary agar plate screening (table 1), an actively cellulolytic culture of Cephalosporium acremonium Corda (C123) was selected for further detailed study. This organism was originally isolated from mouldy hay, and has been deposited in the Commonwealth Mycological Institute collection of fungus cultures by Dr. Lacey of Rothamsted as CMI 96,201.

(b) Growth curves and enzyme synthesis in liquid culture (Fig.1)

Shake flask culture studies of C.acremonium demonstrated that a wide variety of cellulosic substrates ranging in complexity from glucose to native cotton fibres supported the growth of this organism. All these substrates investigated were found to be capable of inducing the synthesis of cell-free enzymes active against cellobiose (β-glucosidase), carboxymethyl cellulose (CMC-ase), acid swollen cellulose (Cx-cellulase) and native cotton (cellulase). With glucose grown cells however, the liberation of significant quantities of these enzymes only became apparent during the stationary and senescent phases of growth, a phenomenon also noted with the CMC-ase and Cx-cellulase of cellobiose grown cells. Although a similar increase in the titre of β-glucosidase was noted at the onset of the stationary phase of cellobiose grown cells, the induction of β-glucosidase by this substrate was pronounced at an early stage of growth. Growth on CMC was sparse, with only a slight initial increase in cell material being measured; synthesis of Cx enzymes although limited was detectable early in the growth curve. The production of cellulase which could not be detected in normal filtrates

Table 1.

Preliminary screening of organisms for cellulolytic activity.

Culture code.	Organism.	Colony diameter. cm.	Zone diameter. cm.	Extent.
C1	Cephalosporium brotzu	1.08	-	0
C2	C.acremonium Cda ((Gruner))	1.90	1.90	4
C3	C.acremonium Cda ((Koehler))	1.56	1.56	3
C4	C.acremonium Cda ((Janke))	1.38	1.38	2
C5	C.album Preuss	1.25	-	0
C6	C.asteroides griseum Grutzii (Grutz)	1.86	1.86	4
C7	C.ballagii Oomen	1.92	1.92	3
C8	C.carpogenium Ruehle ((Schwartz))	2.10	2.10	3
C11	C.coccidicolum Gueguen ((II))	2.02	2.02	3
C13	C.cretatum Hutchinson	1.89	1.89	1
C14	C.deformans Crandall	3.00	3.00	1
C17	C.lamellaecola Smith	1.42	-	0
C21	C.longisporum Petch	1.34	-	0

C22	C.malorum Kidd	1.95	1.95	4
C23	C.niveolanosum Benedek	2.15	2.15	3
C.24	C.pseudofermentum Cif.	1.95	1.95	2
C25	C.recifei de Leao & Lobo	2.05	2.05	5
C26	C.rubescens Schimon	1.78	1.78	3
C27	C.rubrobrunneum ceribriforme hartmannii Benedek	2.06	2.06	4
C30	C.sclerotigenum M.&F. Moreau ((CBS))	2.43	-	0
C31	C.spinosum Negroni	1.19	-	0
C34	C.serrae Maffei ((Verona)) syn Verticillium serrae (Maffei) v. Beyma	2.20	2.20	0
C35	C.tabacinum v. Beyma	2.37	2.37	3
C36	Verticillium coccorum (Petch) Westerdijk.	2.01	-	0
C37	Barrier cream organism, probably C.sclerotigenum	2.16	2.16	3
C38	Cephalosporium sp.	1.76	1.76	3
C39	Cephalosporium sp	1.74	1.74	2

C40	Cephalosporium sp.	2.17	2.17	5
C42	C.acremonium Cda.	1.70	-	0
C43	Cephalosporium sp.	3.32	3.32	3
C45	C.diospyri Crandall	1.90	1.90	4
C48	C.asteris Dowson from asters at Wisley 1923	1.84	1.84	3
C49	C.eichorniae Padwick from Calpogonium mucunoides Sierra Leone 1951 (F.C. Deighton)	2.00	2.00	4
C51	C.longisporum Petch	2.00	2.00	3
C53	Cephalosporium sp. from bananas, Bermuda 1950 ((J.M. Waterson))	2.19	2.19	5
C54	Cephalosporium sp.	2.00	-	0
C58	Cephalosporium sp.	1.90	-	0
C59	Cephalosporium sp.	1.46	-	0
C60	Cephalosporium sp.	Irregular	-	0
C61	C.asteris from NCTC March 1944	2.20	2.20	5
C62	C.stuehmeri	0.56	-	0

C64	C.subverticillatum	Irregular	-	0
C65	Cephalosporium sp. from soil 1949	-	-	0
C66	C.acremonium	-	-	0
C67	Cephalosporium sp. from oat root surface 1949	1.12	1.12	1
C68	Cephalosporium sp. Culture contaminant Kew 1951 H.A.Dade.	1.89	1.89	4
C70	Gliomastix convoluta	2.16	2.16	5
C71	G.convoluta v. felina	2.15	2.15	2
C72	G.convoluta	Irregular	Irregular	5
C73	Verticillium dahliae	-	-	0
C77	Tilachlidium tomentosum strain Truter (Schrad) Lindeau	0.80	-	0
C86	C.potronii((Vuill)) Oomen	0.80	-	0
C88	Hyalopus onychophilus (Vuill) Aschieri	Irregular	-	0
C89	Gliomastix convoluta	0.90	-	0

C93	Cephalosporium sp.	1.15	1.15	3
C94	Cephalosporium sp.	-	-	0
C96	Cephalosporium sp	Irregular	-	0
C99	Cephalosporium sp.	1.40	-	0
C100	Cephalosporium sp.	3.50	-	0
C102	Tilachlidium sp.	1.80	1.80	5
C103	Tilachlidium sp.	2.05	2.05	4
C104	Cephalosporium sp. soil at Nailsea.	2.94	2.94	4
C106	C.pammelii	4.60	4.60	1
C110	Cephalosporium sp. Soil at Weston-Super-Mare.	Irregular	-	0
C111	Cephalosporium sp. Soil at Weston-Super-Mare.	2.80	2.80	2
C113	C.pammelii Soil at Weston-Super-Mare.	Irregular	-	0
C114	C.roseo griseum Agr. Res. Inst. New Delhi	Irregular	-	0
C119	Emericellopsis minima Stolk	Irregular	-	0

C123	Cephalosporium acremonium Corda. Lacey from mouldy hay, 1959.	2.05	2.05	5
C138	C.gramineum	0.87	-	0
C140	Cephalosporium sp.	1.43	-	0
C142	Cephalosporium sp.	2.23	2.23	3
C143	C.madurae Padhye-Sukapure & Thirum	2.40	2.40	4
C144	C.aphidicola Petch.	2.25	-	0
C145	C.incarnatum Sukapure & Thirum	2.04	2.04	4
C146	C.acremonium Corda	1.70	1.70	3
C150	C.crotocinigenum Schol-Schwartz	2.40	2.40	2
C152	C.acremonium Corda	2.23	2.23	4

prepared from CMC cultures was confirmed by assaying filtrates concentrated ten-fold by freeze drying. Assuming no loss of activity during the preparation of these concentrated extracts, the assay results indicated cellulase to be present in the original filtrates at levels varying between 0.5 units/ml after 3 days incubation to 2.0 u/ml after 6 days. Growth of C.acremonium on the acid swollen cellulose substrate was accompanied by a steady increase in the titre of Cx enzymes over the first 5 days; cellulase activity was not detected until after 5 days growth. Although no attempt was made to establish a growth curve for the organism on this substrate due to the presence of varying amounts of residual cellulose, growth was well supported and microscopical examinations of the cultures revealed almost complete solubilization of the substrate after 6-7 days incubation. In view of the short incubation period required for the synthesis of high yields of Cx enzymes, acid swollen cellulose was used routinely for the production of these components.

Examinations of enzyme production in cultures grown on native cotton boll fibres and cotton wool over periods extending to 20 days revealed almost identical synthesis patterns. After rapid initial production of Cx activities, titres of these enzymes increased steadily for a further 12-13 days, cellulase activity becoming detectable after 4-5 days incubation. A slight increase in the rate of enzyme liberation after about 14 days was followed by a period in which the rate of production of all enzymes decreased and in which there appeared to be some destruction of the Cx-cellulase. Consequently during routine enzyme production cultures were harvested after 15-16 days incubation. It was also noted that the phases described above coincided closely with

Figure 1.

Growth curves and enzyme synthesis.

a) Glucose grown cells.

△ - Dry weight

● - β-glucosidase

□ - CMC-ase

■ - Cx-cellulase

○ - Cellulase

b) Cellobiose grown cells.

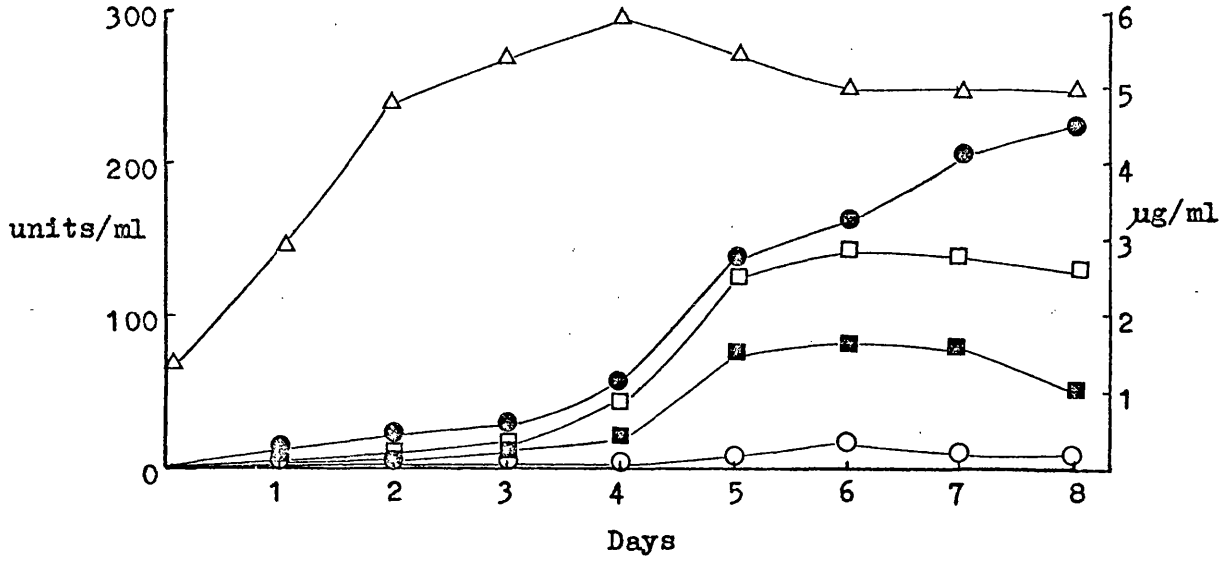
△ - Dry weight

● - β-glucosidase

□ - CMC-ase

■ - Cx-cellulase

○ - Cellulase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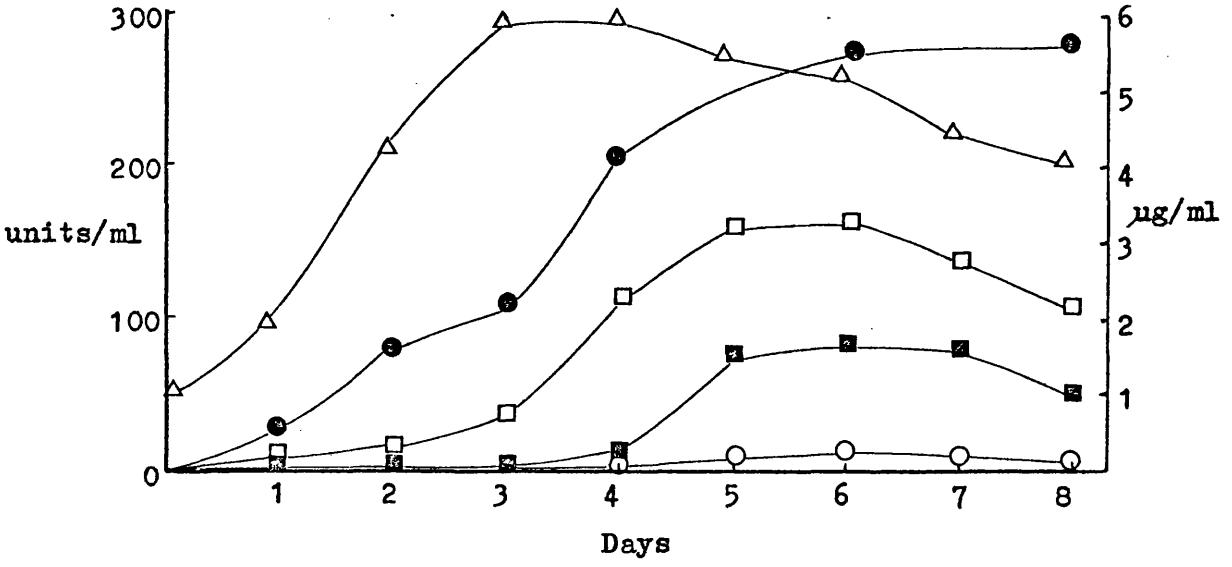
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Days

Figure 1 (cont).

c) CMC grown cells.

△- Dry weight

●- β-glucosidase

□- CMC-ase

■- Cx-cellulase

○- Cellulase

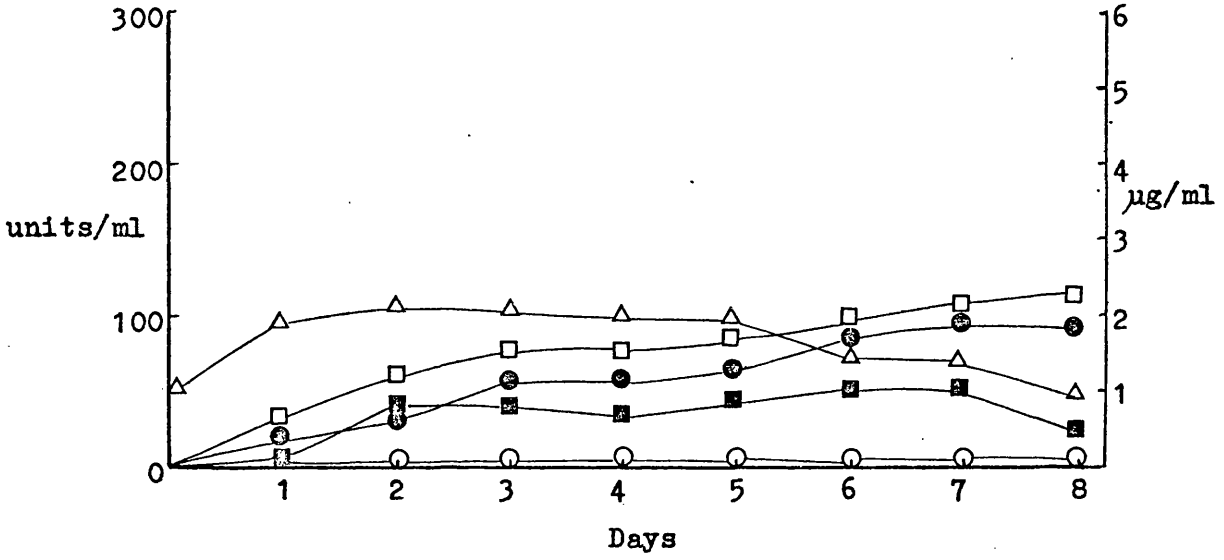
d) Cells grown on acid swollen ball-milled cellulose.

●- β-glucosidase

□- CMC-ase

■- Cx-cellulase

○- Cellulase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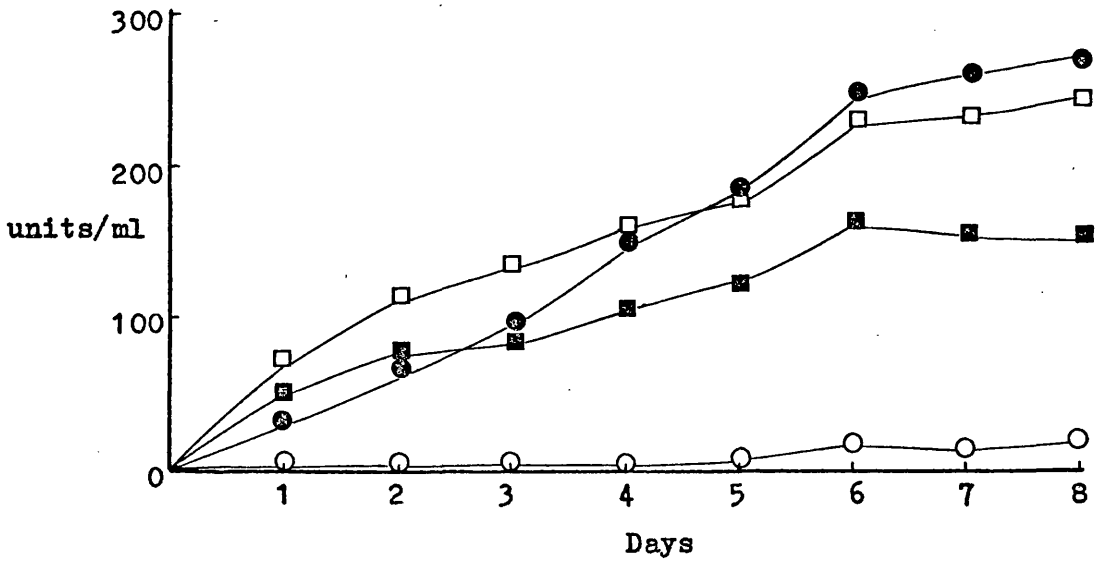
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units/ml
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6
7
8
Days

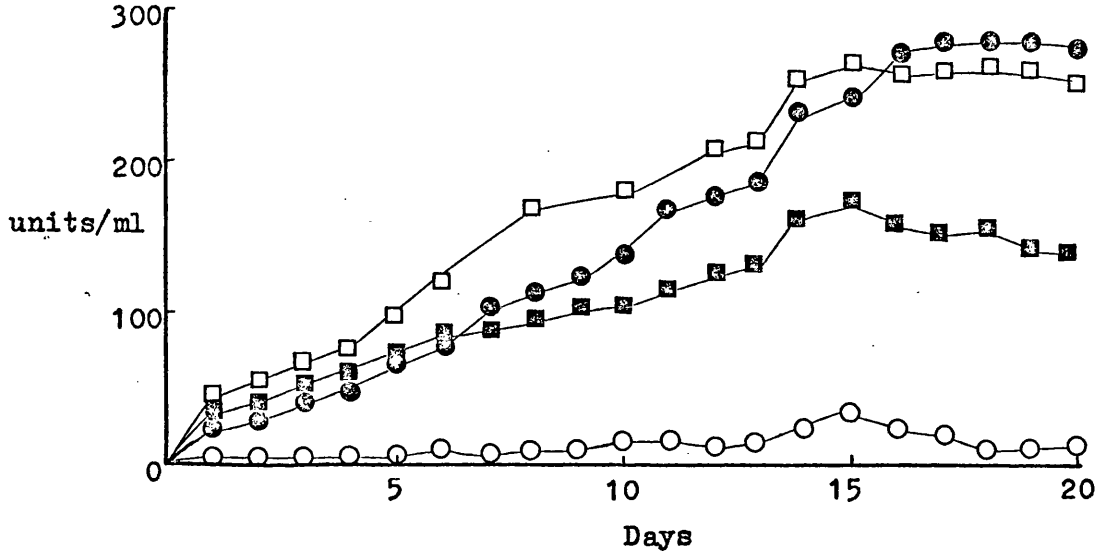
Figure 1 (cont).

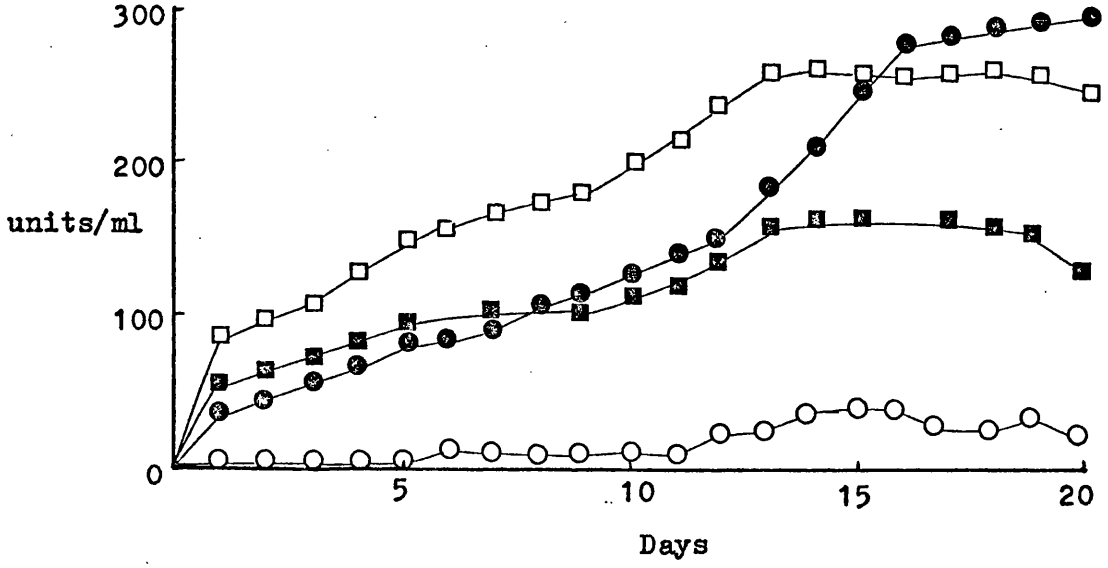
e) Cells grown on dried cotton boll fibres.

- ● - β-glucosidase
- □ - CMC-ase
- ■ - Cx-cellulase
- ○ - Cellulase

f) Cells grown on non-absorbent cotton wool.

- ● - β-glucosidase
- □ - CMC-ase
- ■ - Cx-cellulase
- ○ - Cellulase


300
200
100
0
units/ml
5
10
15
20
Days


300
200
100
0
units/ml
5
10
15
20
Days

macroscopic changes occuring in the cultures. After incubation for 24h the turbidity of the free liquid in the cultures was found to have decreased considerably, and microscopical examinations revealed that most of the cells introduced in the inoculum had accumulated within the fibrous matrix of the cellulose substrate. Growth of the organism during the next 5-6 days was confined to this matrix, assesment of growth being considerably assisted by the development of cell pigmentation. Fragmentation of the substrate during this period was either absent or very limited in extent (plate 2 (2)). The second week of incubation was however characterized by a rapid process of fibre fragmentation with the liberation of numerous short fibres ranging in size from 20 μ x 10 μ to 1000 μ x 14 μ, (plates 2 (3), 2 (4), 3, 4, 5). Although an increase in enzyme titres was measured at about the time of completion of this fragmentation process, no further solubilization of the short fibres was observed despite extended incubation of some cultures for periods up to 6 weeks. This observation was investigated further by obtaining purified samples of the fibres on grade 1 porosity glass sinters. Under these conditions the bulk of the cells passed through the sinter leaving a residue consisting predominantly of the short cellulose fibres. After thorough washing with distilled water the fibres were freeze dried and reincorporated as carbon source into fresh Skinner's medium which was subsequently inoculated and incubated as previously described. Growth of the organism was sparse, and no further solubilization of the short fibres was observed over a period of 6 weeks. The fibres were however completely hydrolysed within 10 days when incorporated as carbon source into Selby & Maitland's (1967) medium and

Plate 1.

Small scale disc electrophoresis gels.

Left, general protein stain;

Right, location of β-glucosidase band.

Plate 2.

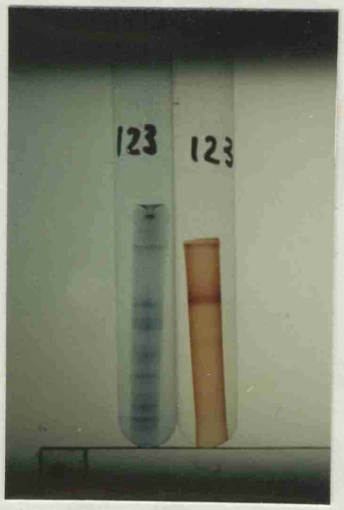
Stages of growth of C.acremonium on native cotton fibres.

1) Uninoculated control.

2) 6 day culture.

3) 12 day culture.

4) 18 day culture.


123
123


1
2
3
4

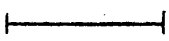
Plate 3.

Undegraded native cotton fibres.

10 μ

Plate 4.

Partially degraded cotton fibre from 12 day culture.



10 μ

Plate 5.

Residual short cotton fibre from 18 day culture.

10 μ

inoculated with the same culture of Trichoderma viride (CMI 92, 027) investigated by these workers. Although this medium was found to support slightly better growth of C.acremonium than Skinner's medium, this was believed to be due to the inclusion of malt, yeast extract and distillers solubles in the former. Cultures of C.acremonium however remained incapable of solubilizing residual short fibres in Selby & Maitland's medium.

Quantitative measurements indicated dried cotton boll fibres and cotton wool to be equally susceptible to enzymic hydrolysis, the residual fibres obtained from cultures containing these substrates comprising respectively 52% and 49% w/w of the original fibrous material. These values may however be high due to slight contamination with fungus.

(c) Solubilization of cell bound enzymes (table 2).

As preliminary examinations had revealed that cell fragments of C.acremonium possessed appreciable Cx enzyme activities, more detailed estimations showed that 1g of freeze dried material contained approximately 12,000 units of β-glucosidase, 15,000 units of CMC-ase and 9,000 units of Cx cellulase. Although cellulase activity was absent from the insoluble cell fragments, slight activity was detected in the supernatant fractions of two solubilization treatments. Cx enzymes were found to be fairly easily removed from the cell fragments by a variety of treatments with the exception of 8M urea which not surprisingly produced complete denaturation. In all other treatments with the exception of β-glucosidase solubilized by calcium chloride, the total activity of each enzyme measured after treatment was found to be greater than that measured originally, indicating higher activity in solution than when cell-bound. The calculated total

Solubilization of cell-bound enzymes (Table 2).

Treatment		Total units β-glucosidase	% change	Total units CMC-ase	% change	Total units Cx-cellulase	% change	Total units cellulase	% change
None		1220	-	1520	-	900	-	nil	-
NaCl	supernatant	1100	+26%	1150	+26%	1650	+109%	250	-
	cell debris	440		770		230		nil	
$CaCl_2$	supernatant	500	-28%	400	+2%	250	+11%	nil	-
	cell debris	380		1150		750		nil	
Urea	supernatant	nil	-	nil	-	nil	-	nil	-
	cell debris	nil		nil		nil		nil	
H_2O	supernatant	1150	+37%	550	+8%	440	+10%	nil	-
	cell debris	530		1100		550		nil	
Citrate	supernatant	900	+21%	950	+23%	400	+30%	nil	-
	cell debris	580		920		770		nil	
Strep. enzyme	supernatant	780	+9%	1130	+30%	1220	+78%	180	-
	cell debris	550		850		380		nil	

percentage changes in activity following treatment take into account not only increases in activity due to solubilization but also any simultaneous loss of activity due to enzyme denaturation, the latter being apparently more pronounced than the former in the case of β-glucosidase treated with calcium chloride. As cells with no prior solubilization treatment were assayed in citrate buffer, it is interesting to note that an increase in activities was observed in cells which had been previously exposed to this buffer before assay, and also in cells previously exposed to water. With hindsight, this increase is apparently a function of the double elution by water or buffer as opposed to a single opportunity for elution when cell fragments are assayed direct. It is also to be expected that the elution effect which occurs in the assay system may more effectively remove enzyme from the cell surface in the presence of substrate to which the enzyme could be bound, thus disturbing the equilibrium of the system.

These results indicated β-glucosidase to be least affected by solubilization, followed in order of increased activity by CMC-ase and Cx-cellulase. This order was however reversed in the case of cell fragments treated with distilled water. Considerable denaturation of enzymes suspended in distilled water at room temperature may however be expected, and this reversal may consequently be interpreted as indicative of the relative stabilities of the Cx components.

In view of the efficient action of citrate buffer in releasing cell-bound Cx enzymes, the possibility that the buffer used for washing intact cells and cell fragments during harvesting had also released any cell-bound C1 component was investigated

by assaying samples of the washings for cellulolytic activity. No evidence for liberation of any components other than β-glucosidase, CMC-ase and Cx-cellulase was obtained, and in contrast to the extensive solubilization measured at room temperature, only slight release of Cx enzymes occured during centrifugation at 0^{o} in citrate buffer.

(d) Enzyme velocity measurements (fig.2).

Velocity-time curves of cell-free and cell-bound Cx enzyme components followed the general form of most enzymic reactions in which the velocity falls with time. The periods during which the rates of hydrolysis were observed to be linear were approximately the initial 50 min of incubation in the case of β-glucosidase and the initial 4h in the case of CMC-ase and Cx-cellulase under the assay conditions described. Although the cellulase curve was rather irregular with no reaction being measurable during the first three days, the rate of hydrolysis of unmodified cotton fibres by cell-free extracts was regarded as linear for the initial 8 days of incubation. It is unlikely that the sudden appearance of cellulase activity after 3 days was due to contamination of incubation mixtures as the system contained sodium azide as an antimicrobial agent.

Before adopting the standard incubation periods which fell well within these limits of linear action, the various causes contributing to the subsequent decline in reaction rates were investigated. Although product inhibition is often regarded as the main factor affecting velocity in this manner, similar effects may be produced if the degree of saturation of the enzyme with substrate falls due to the decrease in substrate concentration, or if the enzyme or a co-enzyme undergoes denaturation during

Figure 2.

Enzyme velocity measurements.

a) Action on ONPG.

● - Culture filtrate and cell fragments

△ - Culture filtrate

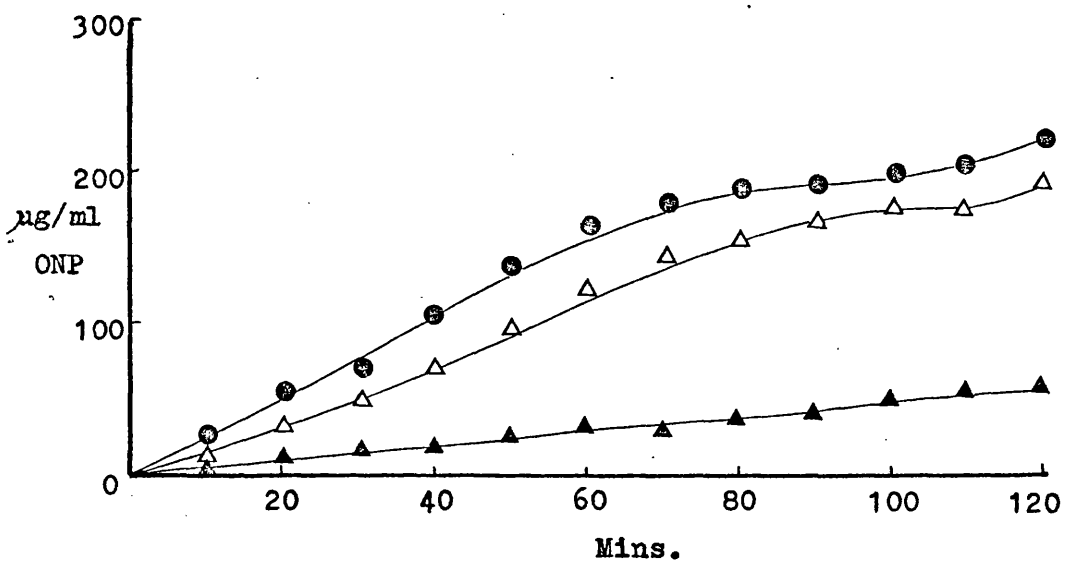
▲ - Cell fragments

b) Action on CMC.

● - Culture filtrate and cell fragments

△ - Culture filtrate

▲ - Cell fragments


300
200
µg/ml
ONP
100
0
20
40
60
80
100
120
Mins.

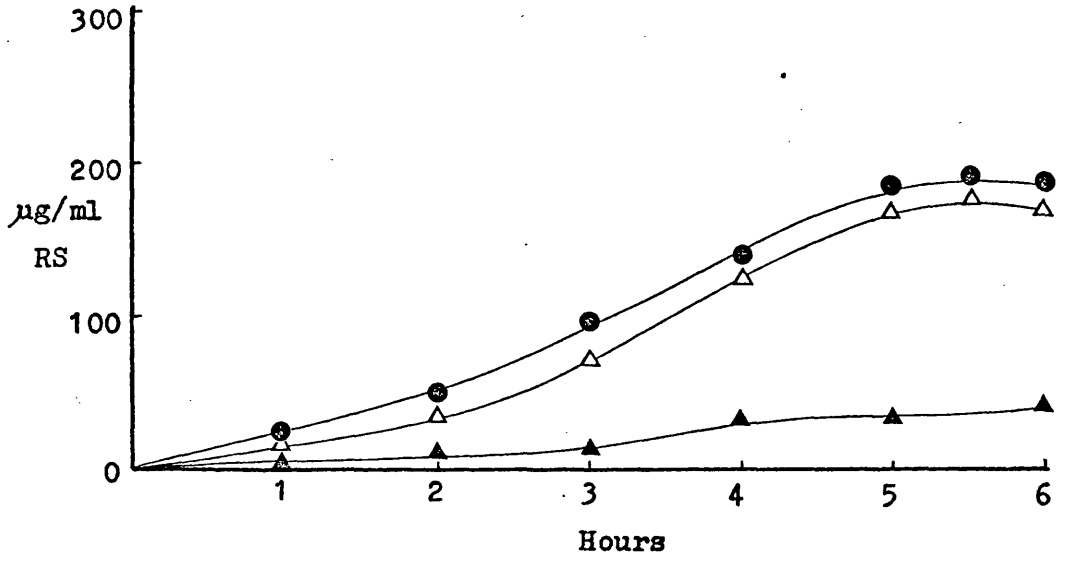

300
200
µg/ml
RS
100
0
1
2
3
4
5
6
Hours

Figure 2 (cont).

c) Action on acid swollen ball-milled cellulose.

● - Culture filtrate and cell fragments

△ - Culture filtrate

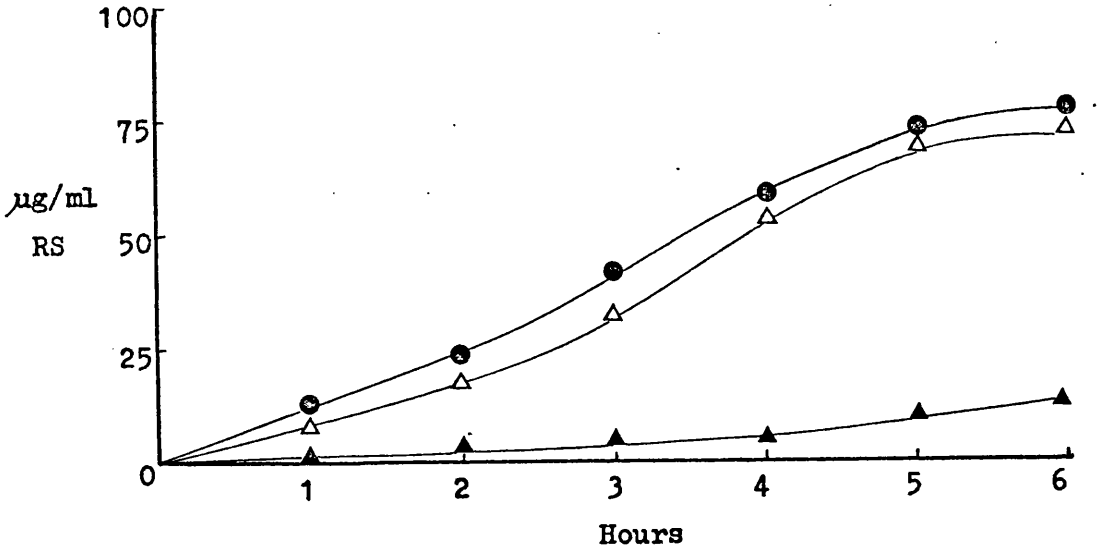
▲ - Cell fragments

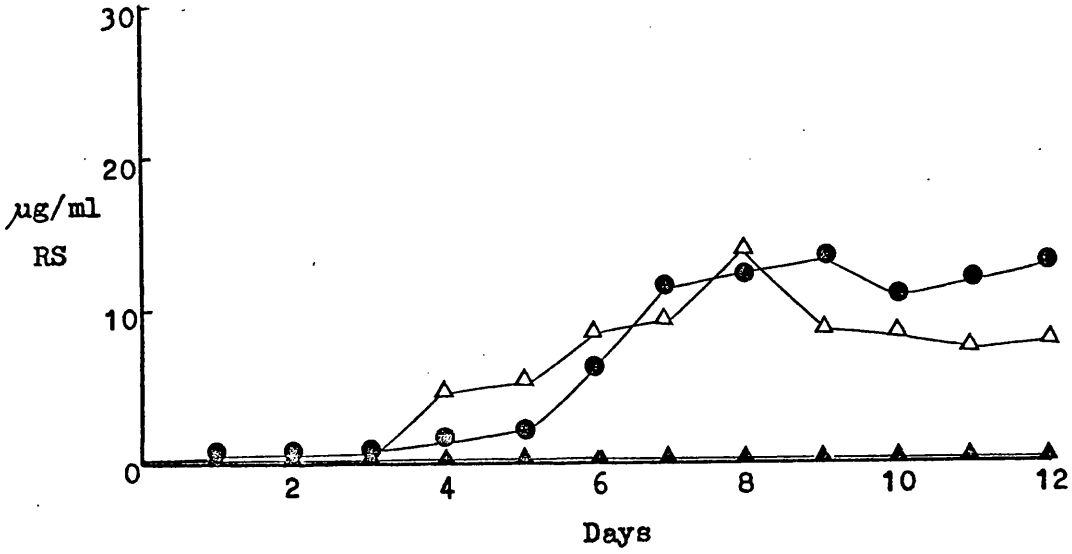
d) Action on cotton fibres.

● - Culture filtrate and cell fragments

△ - Culture filtrate

▲ - Cell fragments


100
75
µg/ml
RS
50
25
0
1
2
3
4
5
6
Hours


30
20
µg/ml
RS
10
0
2
4
6
8
10
12
Days

incubation. As all of these factors may affect the velocity simultaneously, it is only during the early stages of attack when they have not had time to operate that assay conditions are accurately known. Because detailed analyses of enzyme velocity curves require the use of isolated and purified enzymes, simple confirmation of constant enzyme saturation was obtained at this stage of the investigation by incubating equal volumes of an enzyme extract with varying concentrations of substrate. Substrate concentrations beyond which no further increase in reaction rates were noted using freshly prepared unconcentrated extracts were found to be approximately 5×10^{-4} molar ONPG, 0.25% w/v CMC and 0.5% w/v acid swollen cellulose. Although the final concentration of ONPG used in routine assays was thus approximately five times the saturating concentration required by freshly prepared extracts, the CMC and acid swollen cellulose preparations provided lower margins of safety as the final concentrations of these substrates in standard assay mixtures were only approximately twice the minimum required. In view however of the difficulties encountered in handling more concentrated preparations of these substrates it was found to be more practicable when examining purified extracts of high activity to assay a diluted sample of the enzyme, thereby ensuring constant enzyme saturation.

Although the contribution of degree of saturation to the eventual decline in reaction rates of Cx components was thus considered negligible, the situation was however found to be more complex with cellulase activity. Each increase in the concentration of cotton fibres in incubation mixtures from 0.5% to 5.0% w/v produced a corresponding increase in reaction rate

until the point was reached when it became impracticable to increase the substrate concentration further due to the total absorption of the liquid phase of the reaction mixtures by the fibres. In this situation however, dilution of the enzyme in an effort to produce saturation conditions resulted in a level of activity too low to be reliably detected. The 5.0% concentration of cotton fibres used in routine cellulase estimations was thus the highest level practicable, resulting in an unavoidable fall in the degree of saturation during incubation.

The extent of enzyme denaturation was assessed initially by assaying enzyme preparations which had undergone previous incubation at 40° in citrate buffer pH 5.0 for varying periods in the absence of substrate. No denaturation of Cx components was noted during the standard incubation periods (Table 3) although loss of activity was apparent after prolonged incubation for up to 12 days. Cx enzyme stabilities varied considerably over this period, β-glucosidase retaining approximately 74% of its original activity after 12 days, CMC-ase 18% and Cx-cellulase being rendered totally inactive after 8 days. Cellulase activity however decreased gradually during the standard assay period of 7 days and was not detected after 8 days. As the presence of substrate may exhibit a stabilizing effect on an enzyme, a more accurate check on the extent of denaturation was made while investigating the extent of product inhibition.

The effect of accumulated product on enzyme velocity was assessed by subjecting samples of reaction mixtures which had attained the plateau region of the velocity-time curve to fractionation on 1.0 x 20.0 cm columns of Sephadex G25 equilibrated with citrate buffer pH 5.0. Elution with buffer at 4°

Loss of Enzyme Activity in Culture Filtrate Stored at 40°

in the Absence of Substrate in Citrate Buffer pH 5.0

(Table 3).

Enzyme	% of activity remaining after:																		
	mins						hours					days							
	0	20	40	60	80	100	1	2	3	4	5	1	2	3	4	5	8	10	12
β-glucosidase 287 u/ml	100	96	97	101	97	100	nt	nt	nt	nt	nt	100	100	101	102	92	90	86	74
CMC-ase 220 u/ml	100	nt	nt	nt	nt	nt	99	104	103	100	100	95	86	91	82	70	66	43	18
Cx-cellulase 150 u/ml	100	nt	nt	nt	nt	nt	106	99	103	101	100	100	65	43	33	33	18	0	0
Cellulase 18 u/ml	100	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	77	61	66	61	50	28	0	0

(nt = not tested)

Product Inhibition Studies (Table 4).

Initial conc. of enzyme in mixture	Incubation period to attain plateau region	Conc. of product	Volume applied to Sephadex G25	Volume recovered	Reconstituted volume after lyophilization	Units of enzyme recovered	% activity restored
B-glucosidase 32 u/ml	120 min	215 ug/ml ONP	2.0 ml	12.0 ml	1.0 ml	67	104%
B-glucosidase control	120 min	215 ug/ml ONP	2.0 ml sample diluted to 12.0 ml with buffer		1.0 ml	3	4.7%
CMC-ase 25 u/ml	300 min	190 ug/ml RS	2.0 ml	13.5 ml	1.0 ml	45	90%
CMC-ase control	300 min	190 ug/ml RS	2.0 ml sample diluted to 13.5 ml with buffer		1.0 ml	nil	nil
Cx-cellulase 17.5 u/ml	360 min	100 ug/ml RS	2.0 ml	13.0 ml	1.0 ml	39	111%
Cx-cellulase control	360 min	100 ug/ml RS	2.0 ml sample diluted to 13.0 ml with buffer		1.0 ml	nil	nil

enabled rapid separation of enzymic components from low molecular weight material to be made. Although all Cx activities were found to be completely restored by this process, cellulase activity was not recovered (Table 4).

Investigations thus indicated that while the decline in Cx activities observed during incubation was due almost entirely to product inhibition, the lack of cellulase activity after 7-8 days incubation was believed to be due mainly to the combined effects of enzyme denaturation and a decrease in the degree of saturation with substrate. In view of the importance of product inhibition in Cx enzyme assays, all routine estimations of β-glucosidase, CMC-ase and Cx-cellulase exhibiting concentrations of product greater than 200 μg/ml ONP, 175 μg/ml and 90 μg/ml of reducing sugar respectively after the standard incubation periods were re-assayed using appropriately diluted enzyme preparations.

(e) Viscometry (fig.3).

Viscometric studies of the enzymic hydrolysis of CMC demonstrated this method to be an extremely sensitive and rapid means of detecting CMC-ase activity. While appreciable quantities of reducing sugars were only detected after lengthy incubation periods, a rapid drop in viscosity was measured during the first 10-15 min of incubation followed by a period of gradual decline. Simultaneous measurements of CMC-ase activity by both assay methods revealed that the period of rapid loss of viscosity was associated with a low level of reducing sugar liberation, the latter becoming more pronounced during subsequent incubation. This pattern is characteristic of endoenzymic activity (Wood, 1955), the initial rapid decrease in viscosity being due to

Figure 3.

Viscometry.

Action on CMC of

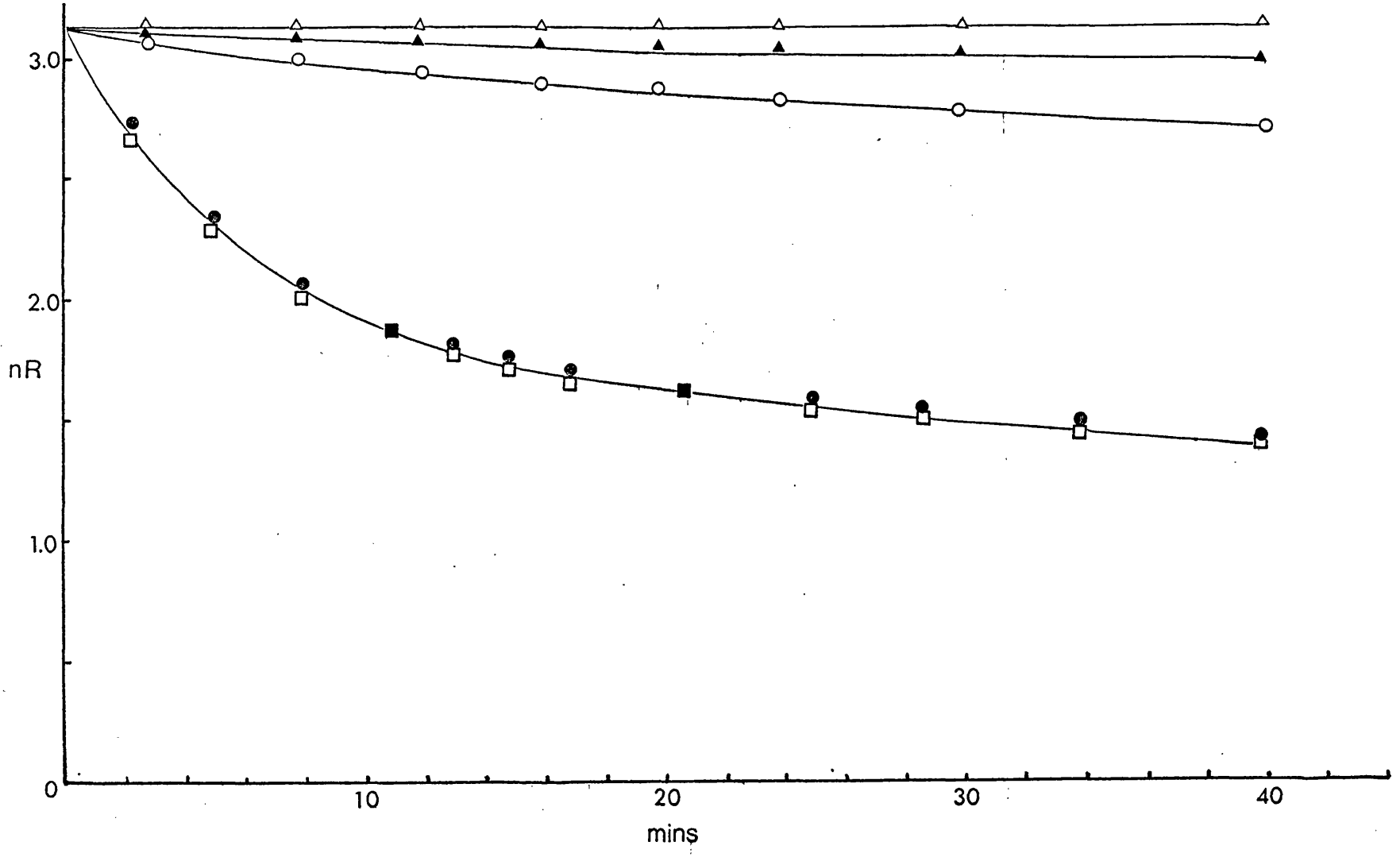
△- Culture filtrate autoclaved at 15 psi.(121°)

○- Culture filtrate heated for 10 min at 100°.

▲- Culture filtrate heated for 10 min at 100° and inhibited with gluconolactone.

□- Unheated culture filtrate.

●- Unheated culture filtrate inhibited with gluconolactone.


3.0
2.0
1.0
0
nR
10
20
30
40
mins

random cleavage of the longer chain components of the substrate, and the lack of reducing power the result of a low ratio of terminal reducing groups to degree of polymerization. While having little effect on viscosity, subsequent hydrolysis of the shorter substrate molecules showed a marked increase in reducing power due to a high terminal reducing group:DP ratio.

At an early stage of this investigation before isolated components of the cellulase system of C.acremonium had been obtained, evidence of "overlapping" action between two suspected components was discovered during viscometry studies. The control incubation mixture which normally contained an autoclaved sample of culture filtrate was prepared on one occasion using a filtrate denatured by heating at 100° for 10 min, and was found to produce a slight but steady loss of viscosity which could only be prevented by increasing the duration of heating to 30 min at 100°. Accurate measurements of this thermostable activity revealed that what at first appeared to be a linear decrease in viscosity indicating exo-enzymic activity was in reality a flattened curve, suggesting either an extremely low level of residual CMC-ase or the action of another enzyme active only on a minor component of CMC. In the latter case the limited decrease in viscosity was believed to implicate low DP substrate molecules which in turn suggested the possible action of β-glucosidase (Reese, Maguire & Parish, 1968; King & Vessal, 1969; Wood,1971). Although no enzyme activity of any description could be detected by standard assay procedures in filtrates heated at 100° for 10 min, subsequent heat denaturation studies confirmed β-glucosidase to be the most thermostable of the Cx components (fig 4).

Further evidence of hydrolysis of CMC by β-glucosidase was

Figure 4.

Selective thermal denaturation.

a) Effect of heating for 1 min on cellulase components of culture filtrate.

● - β-glucosidase

□ - CMC-ase

■ - Cx-cellulase

○ - Cellulase

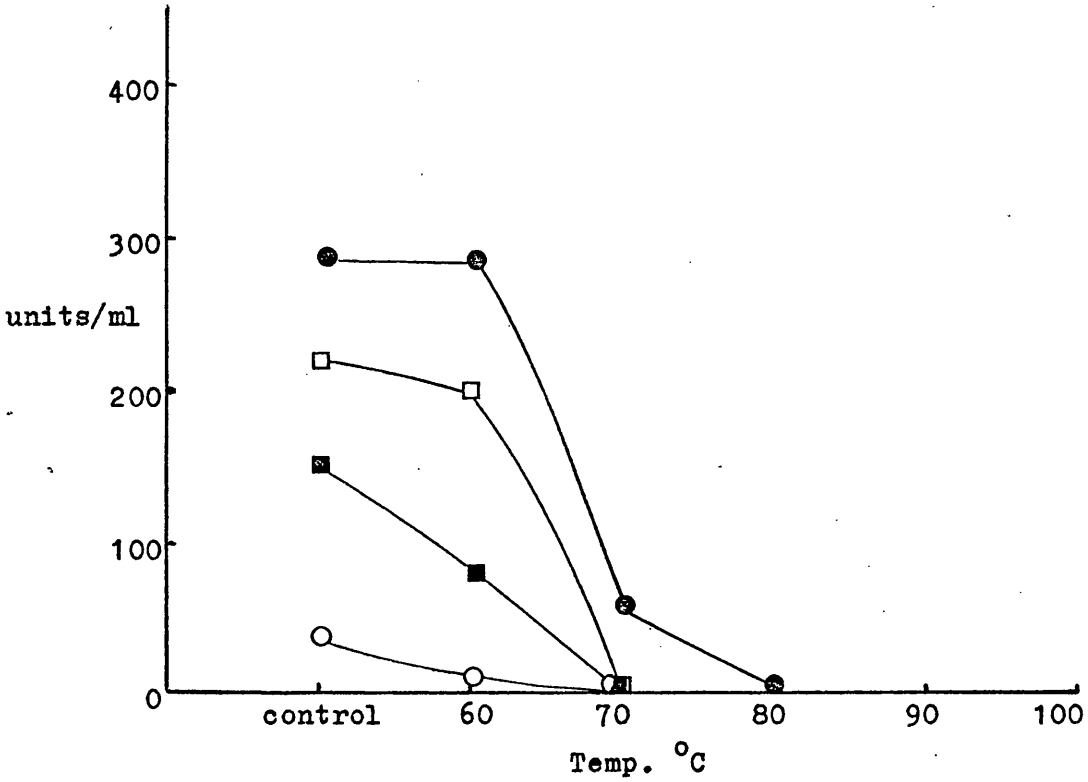
b) Effect of heating at 60° on cellulase components of culture filtrate.

● - β-glucosidase

□ - CMC-ase

■ - Cx-cellulase

○ - Cellulase


400
300
units/ml
200
100
0
control
60
70
80
90
100
Temp. °C

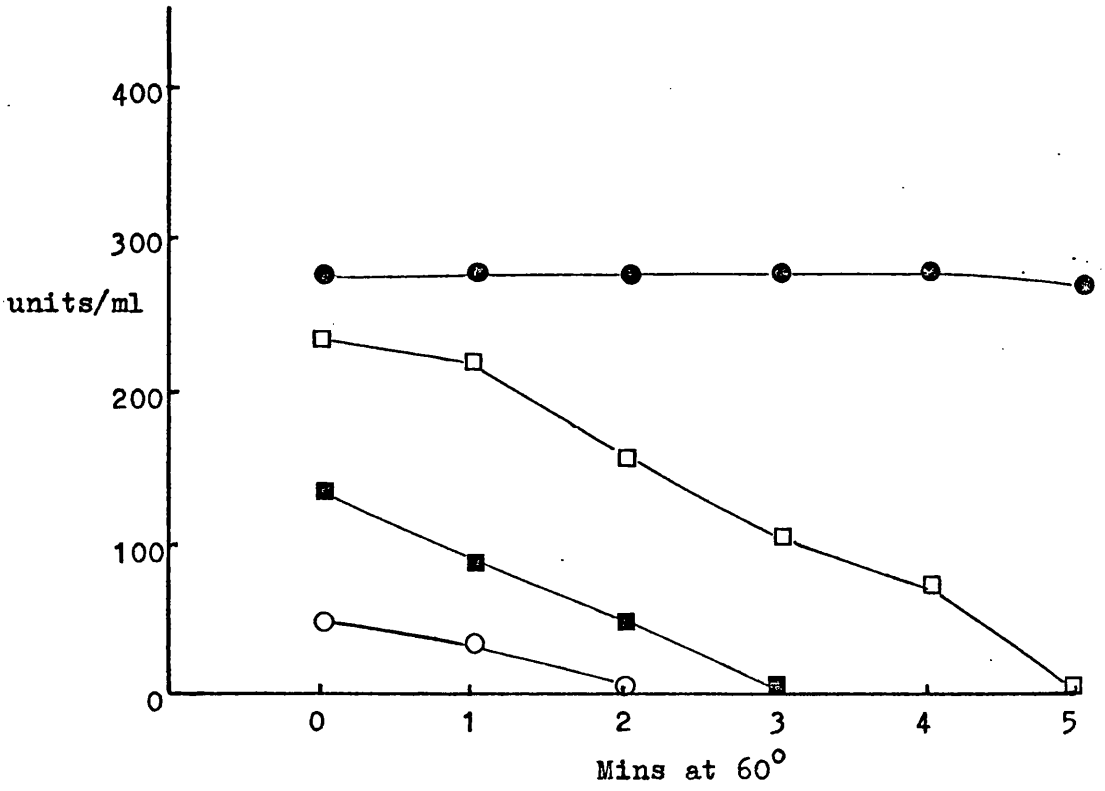

400
300
units/ml
200
100
0
0
1
2
3
4
5
Mins at 60°

Figure 5.

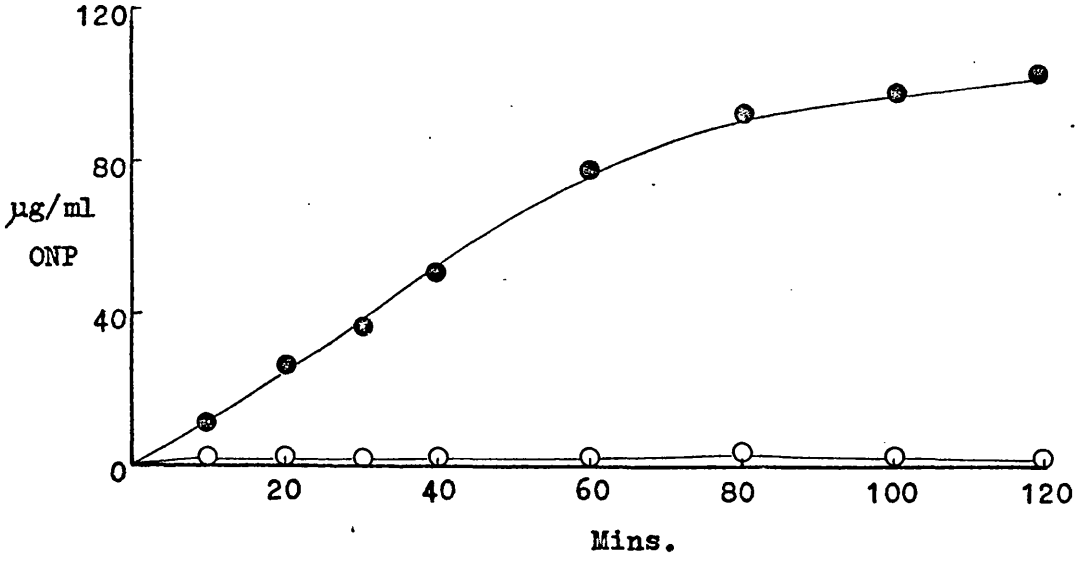
Inhibition of cellulase components in culture filtrate by 1,5-gluconolactone.

a) ● - Hydrolysis of ONPG in uninhibited filtrates.

○ - Hydrolysis of ONPG in inhibited filtrates.

b) ■ - Hydrolysis of CMC in uninhibited filtrates.

□ - Hydrolysis of CMC in inhibited filtrates.


120
80
µg/ml
ONP
40
0
20
40
60
80
100
120
Mins.

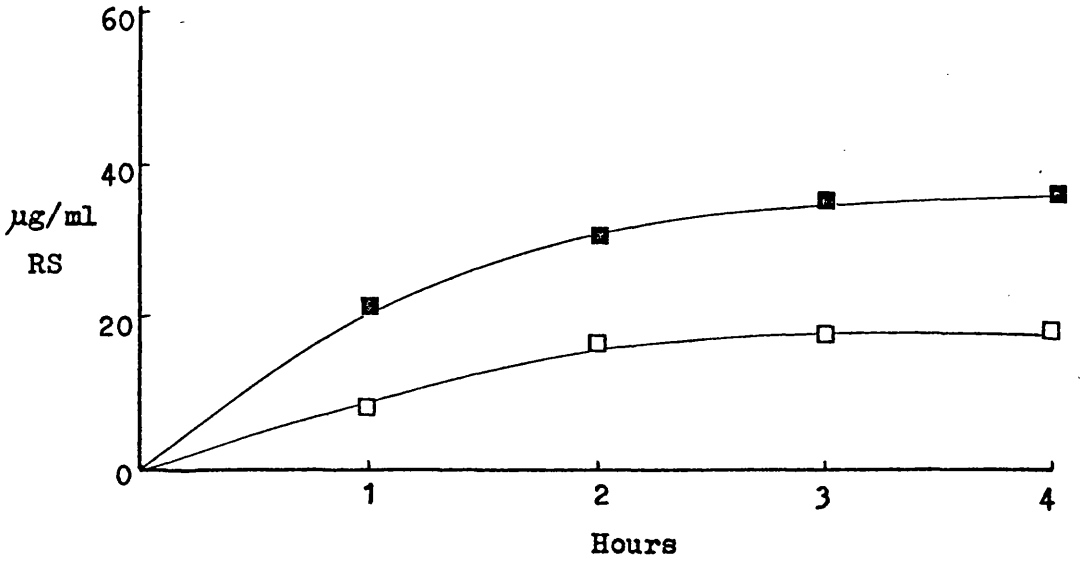

60
40
µg/ml
RS
20
0
1
2
3
4
Hours

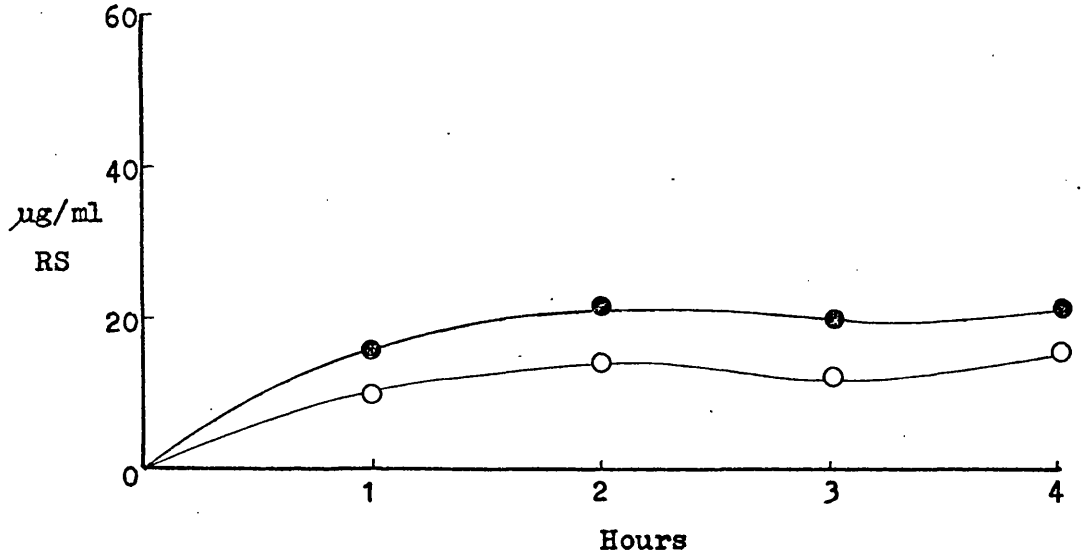
Figure 5 (cont).

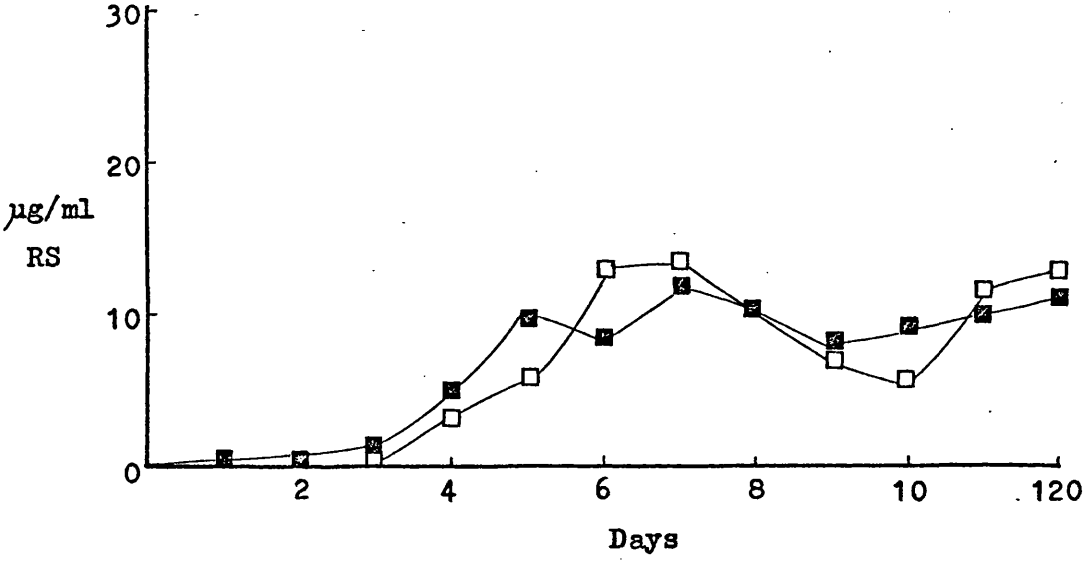
c) ● - Hydrolysis of acid swollen ball-milled cellulose in uninhibited filtrates.

○ - Hydrolysis of acid swollen ball-milled cellulose in inhibited filtrates.

d) ■ - Hydrolysis of cotton fibres in uninhibited filtrates.

□ - Hydrolysis of cotton fibres in inhibited filtrates.


60
40
20
0
µg/ml
RS
1
2
3
4
Hours


30
20
10
0
µg/ml
RS
2
4
6
8
10
120
Days

provided by the discovery that the addition of 1.0 mg/ml of 1,5-gluconolactone to incubation mixtures substantially decreased the rate of hydrolysis by heated culture filtrates but had no measurable effect on unheated filtrates (fig 3). 1,5-gluconolactone is a competitive inhibitor of β-glucosidase and is claimed to be extremely specific, having little or no action on cellulase, CMC-ase or other closely related glucanases, (Jermyn, 1952; Conchie,1954; Reese & Mandels, 1957). While the inhibition of β-glucosidase in unheated filtrates was not detectable by viscometry due to the masking action of the more active CMC-ase component, the inhibitory action of gluconolactone on β-glucosidase was confirmed by measuring the liberation of o-nitrophenol from ONPG in incubation mixtures containing 1.0 mg/ml of the inhibitor, (fig 5). Although viscometry studies indicated no inhibition of CMC-ase by gluconolactone, simultaneous estimations of reducing sugar liberation in identical reaction mixtures revealed an apparent inhibition of this enzyme of approximately 50%. This observation could however be explained by postulating the main product of CMC-ase action to be cellobiose which exhibits an equivalent reducing power of approximately 40% that of glucose, and which would accumulate under conditions of β-glucosidase inhibition. A similar effect was noted by Festenstein (1958) who reported complete inhibition of cellobiase activity in extracts of rumen micro-organisms by 1,4-gluconolactone at a concentration which produced 60% inhibition of CMC-ase as measured by reducing power. Festenstein concluded that this last effect could result solely from the action of the gluconolactone on the cellobiase in the preparation. It is interesting to note that the inhibition was less marked in the case of attack on acid

Figure 6.

Diphasic nature of CMC.

a) Hydrolysis of soluble component of CMC by

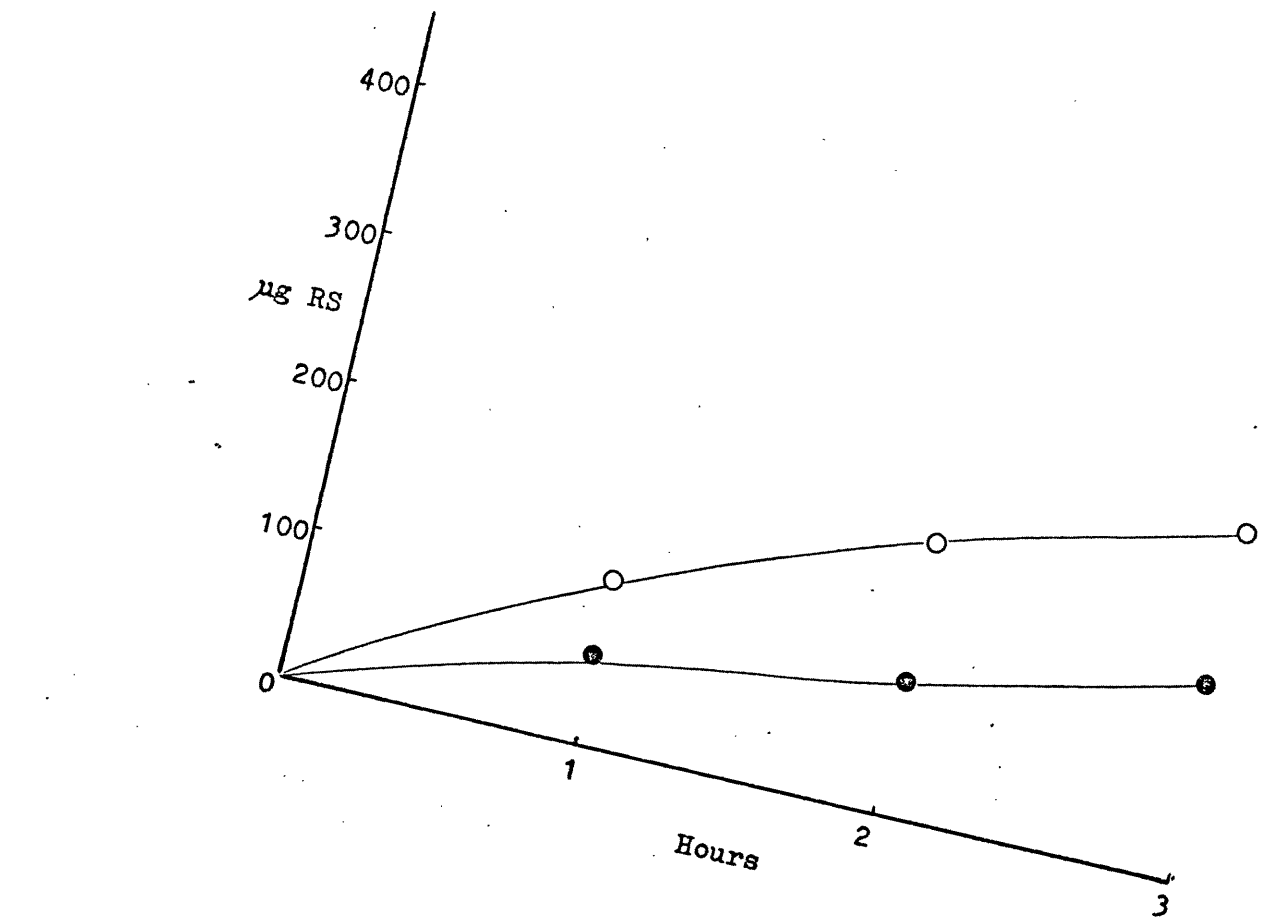
○- Culture filtrate

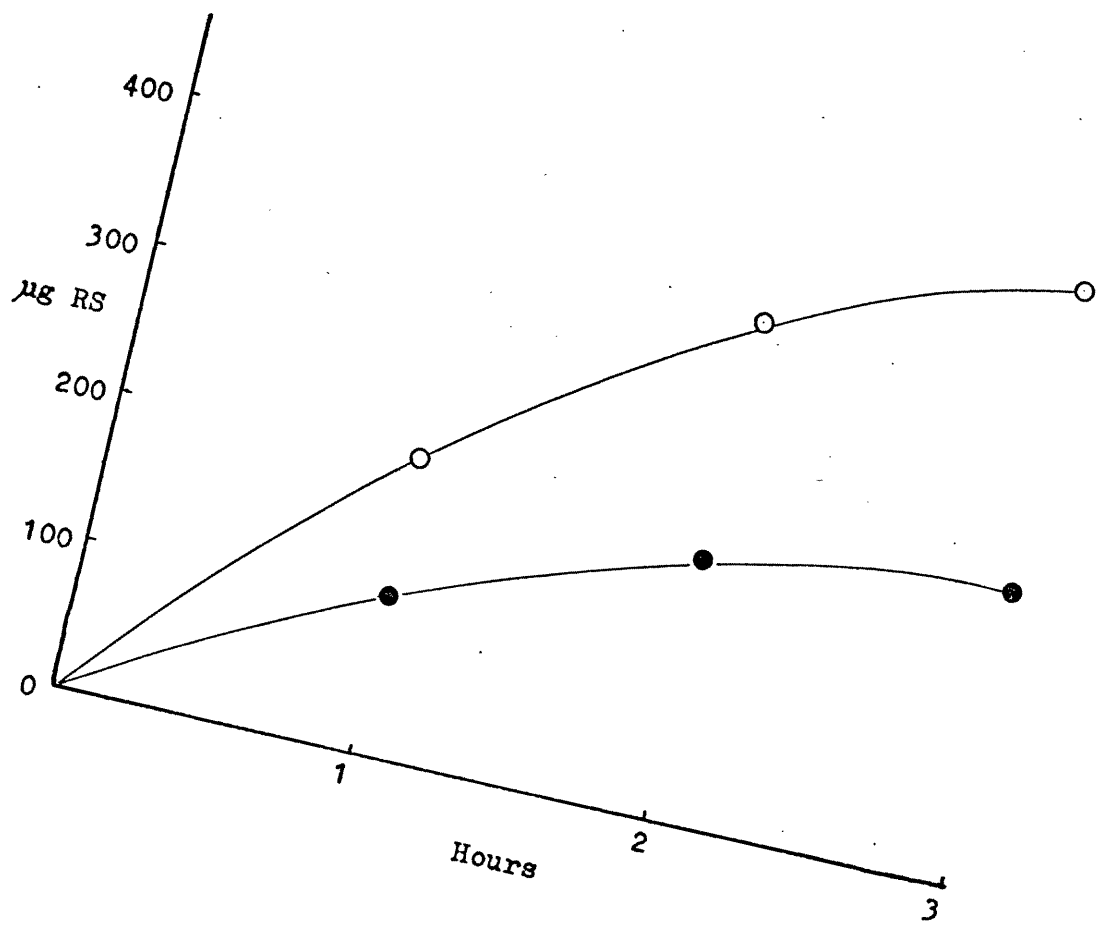
●- Cell fragments

b) Hydrolysis of gel component of CMC by

○ - Culture filtrate

● - Cell fragments


400
300
µg RS
200
100
0
1
2
Hours
3


400
300
µg RS
200
100
0
1
2
Hours
3

swollen cellulose, indicating that cellobiose was probably not the sole or major product of Cx-cellulase action. No inhibition of cellulase activity by 1,5-gluconolactone was noted.

Preliminary studies on the degradation of CMC were completed with an investigation into the diphasic nature of solutions of this substrate. These solutions consisted of insoluble gel particles suspended in a clear viscous liquid component, the particles being readily sedimented by gentle centrifugation into a thick gelatinous pellet. No further solubilization of this gel component was achieved despite repeated applications of either fresh buffer or distilled water at room temperature or 100°. Further investigations demonstrated that uncentrifuged solutions of CMC were more rapidly hydrolysed than centrifuged preparations, indicating the gel component to be more susceptible to enzymic attack than the material in solution. This was confirmed by assaying incubation mixtures containing samples of washed gel as substrate, the extent of hydrolysis of this component being subsequently shown to be approximately twice that of the soluble material when treated with identical cell-free and solubilized cell-bound enzyme extracts (fig 6). Carboxymethyl cellulose as normally prepared is heterogeneous in both degree of substitution (DS) and degree of polymerization (DP), Timell,(1953) believing the former to be more variable than the latter. Although a high degree of substitution results in a more soluble preparation, enzymic activity is curtailed to the extent that when each anhydroglucose unit contains one substituent group, the molecule is resistant to biological attack (Reese, Siu & Levinson, 1950). A low DS renders the cellulose derivative more susceptible to enzymic degradation until this is offset by reduced solubility. The variation of

solubility and susceptibility to attack noted in the CMC preparations used in this investigation could thus be explained on the basis of variations of DS, average estimates measured by the method of Green (1963) being 0.50 for the soluble material and 0.42 for the uncentrifuged preparation. Estimates of the average DS of the gel component were not made due to difficulties experienced in obtaining sufficient dried material for accurate analysis. As suspensions of the gel particles were difficult to handle accurately and could not be used for viscometric studies, the soluble component was chosen as substrate for CMC-ase estimations despite the more resistant nature of this material.

(f) Column chromatography

In contrast to the cellulase systems of most other commonly investigated organisms, fractionation of C.acremonium culture filtrates on Sephadex G75 failed to produce any significant resolution of the enzyme complex, although a marked purification of the cellulolytic components was achieved by their separation from a considerable quantity of non-active protein, pigments and low molecular weight carbohydrate also present in the filtrates (fig 7). Although the elution of β-glucosidase in the void volume indicated a partial separation of this enzyme from the remaining cellulolytic components, the identical elution volumes exhibited by CMC-ase, Cx-cellulase and cellulase with activity peaks coinciding at fraction 9 questioned the concept of these components existing as separate entities. Similarly while all (106%) of the original β-glucosidase was recovered after fractionation, recoveries of CMC-ase, Cx-cellulase and cellulase were 94%, 90% and 90% respectively. Although fractionation on Sephadex G75 was consequently of little value in resolving the enzyme complex

Figure 7.

Fractionation of culture filtrate on Sephadex G75.

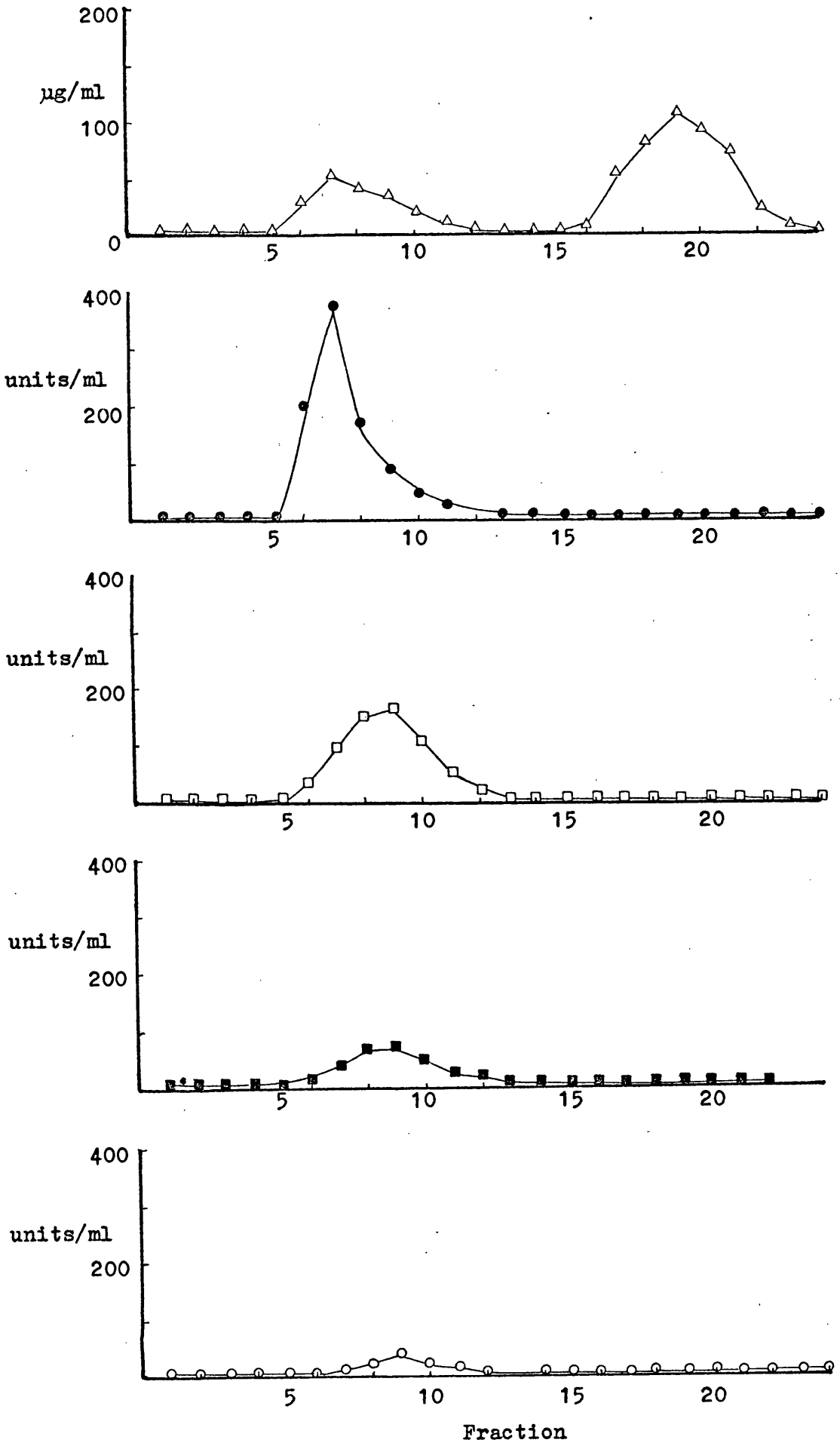
△- Lowry protein

●- β-glucosidase

□- CMC-ase

■- Cx-cellulase

○- Cellulase


200
µg/ml
100
0
5
10
15
20
400
units/ml
200
5
10
15
20
400
units/ml
200
5
10
15
20
400
units/ml
200
5
10
15
20
400
units/ml
200
5
10
15
20
Fraction

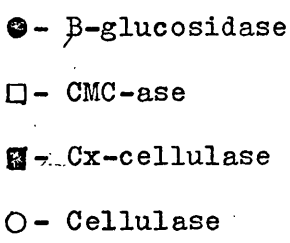
the technique was found to be extremely useful as a preliminary purification process, particularly in the case of extracts subsequently to be studied by gel electrophoresis.

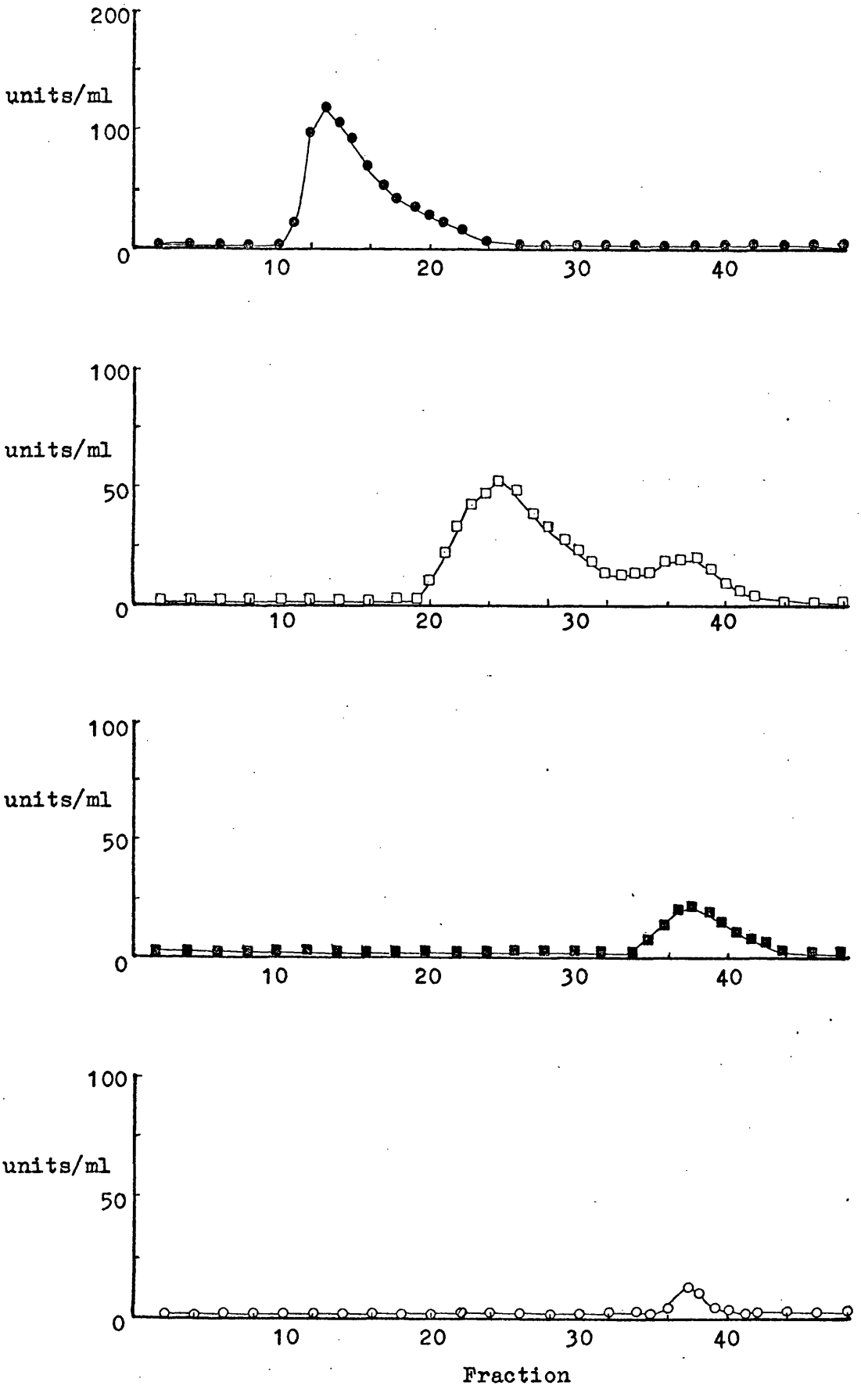
The first evidence of the existence of multienzymic components other than β-glucosidase was provided by fractionation of extracts on Sephadex G200 when a second peak of CMC-ase appeared in the fractions containing the Cx-cellulase and cellulase activities (fig 8). Although β-glucosidase was eluted as previously in the void volume indicating an extremely high molecular weight, separation of this component from the major part of the CMC-ase activity was incomplete. While recovery of β-glucosidase was calculated as 99%, a slight loss of 16% was noted in the case of the total CMC-ase activity. The poor apparent recoveries of Cx-cellulase and cellulase of 42% and 44% respectively which were initially believed to be due to denaturation were subsequently found to be increased to 66% and 62% respectively by the inclusion into these assay mixtures of approximately 50 units/ml of the isolated β-glucosidase component, thereby ensuring that the method of assay which depends on the liberation of reducing groups was in fact measuring glucose, and that the results were not invalidated by the presence of low molecular weight oligomers. Similar additions of β-glucosidase to incubation mixtures containing CMC resulted in an increase of the total CMC-ase activity recovered to 94%, the most significant increase in activity occuring in those fractions containing the high molecular weight CMC-ase component but which were not contaminated by residual β-glucosidase.

In an attempt to improve the resolution of the enzymic components, subsequent fractionations were performed on the Superfine grade of Sephadex G200 at considerably reduced elution

Figure 8.

Fractionation of culture filtrate on Sephadex G200



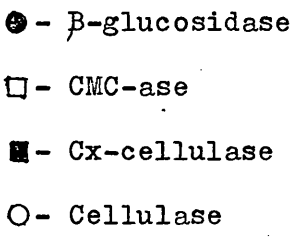

200
units/ml
100
0
10
20
30
40
100
units/ml
50
0
10
20
30
40
100
units/ml
50
0
10
20
30
40
100
units/ml
50
0
10
20
30
40
Fraction

rates. Despite these refinements further separation of activities was found extremely difficult to accomplish, increased resolution only being obtained by elution at the slowest rates practicable (fig 9). The slight losses of Cx-cellulase and cellulase noted previously were however significantly increased by these extended fractionation periods, with recoveries after 96h of 25% and 31% respectively. Re-addition of β-glucosidase raised these figures to 35% and 42% and also increased the total recovery of CMC-ase from 41% to 71%. To obtain an estimate of the rate and extent of denaturation of these components during fractionation, samples of the concentrated extracts normally applied to the columns were diluted 1 in 25 with citrate buffer pH 5.0 and maintained at 4^{o}. At suitable intervals samples were withdrawn for standard assays and the activities expressed as a percentage of those measured initially. The results confirmed that while CMC-ase activity was reduced by only approximately 20% in 100h under these conditions, over 60% of the Cx-cellulase and almost 80% of the cellulase activities were lost (fig 10).

In view of the successful isolation of β-glucosidase from the remaining components and the increased resolution of the two CMC-ase peaks, the lack of any simultaneous separation of Cx-cellulase and cellulase activities and their similar rates of denaturation suggested the possible existence of a single enzymic component eluted in fractions 34-43 (fig 9). This component could thus not only be responsible for the filtrate's activity towards acid swollen cellulose and native fibrous cotton, but also for the second peak of CMC-ase activity. Further evidence supporting this theory was provided by a slight modification of the denaturation analysis described earlier. In this case the

Figure 9.

Fractionation of culture filtrate on Sephadex G200 Superfine at reduced rate of elution.



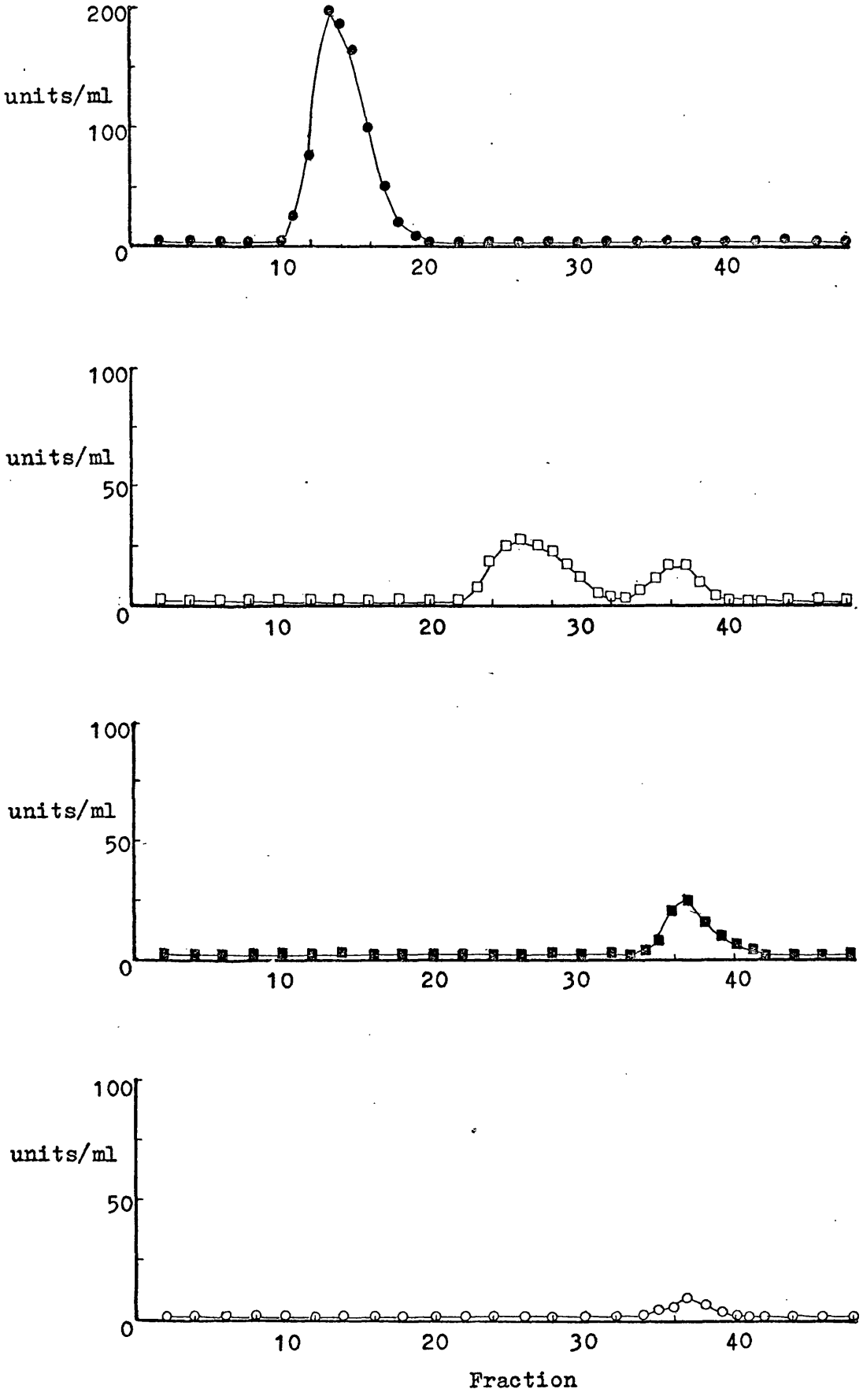

200
units/ml
100
0
10
20
30
40
100
units/ml
50
0
10
20
30
40
100
units/ml
50
0
10
20
30
40
100
units/ml
50
0
10
20
30
40
Fraction

Figure 10.

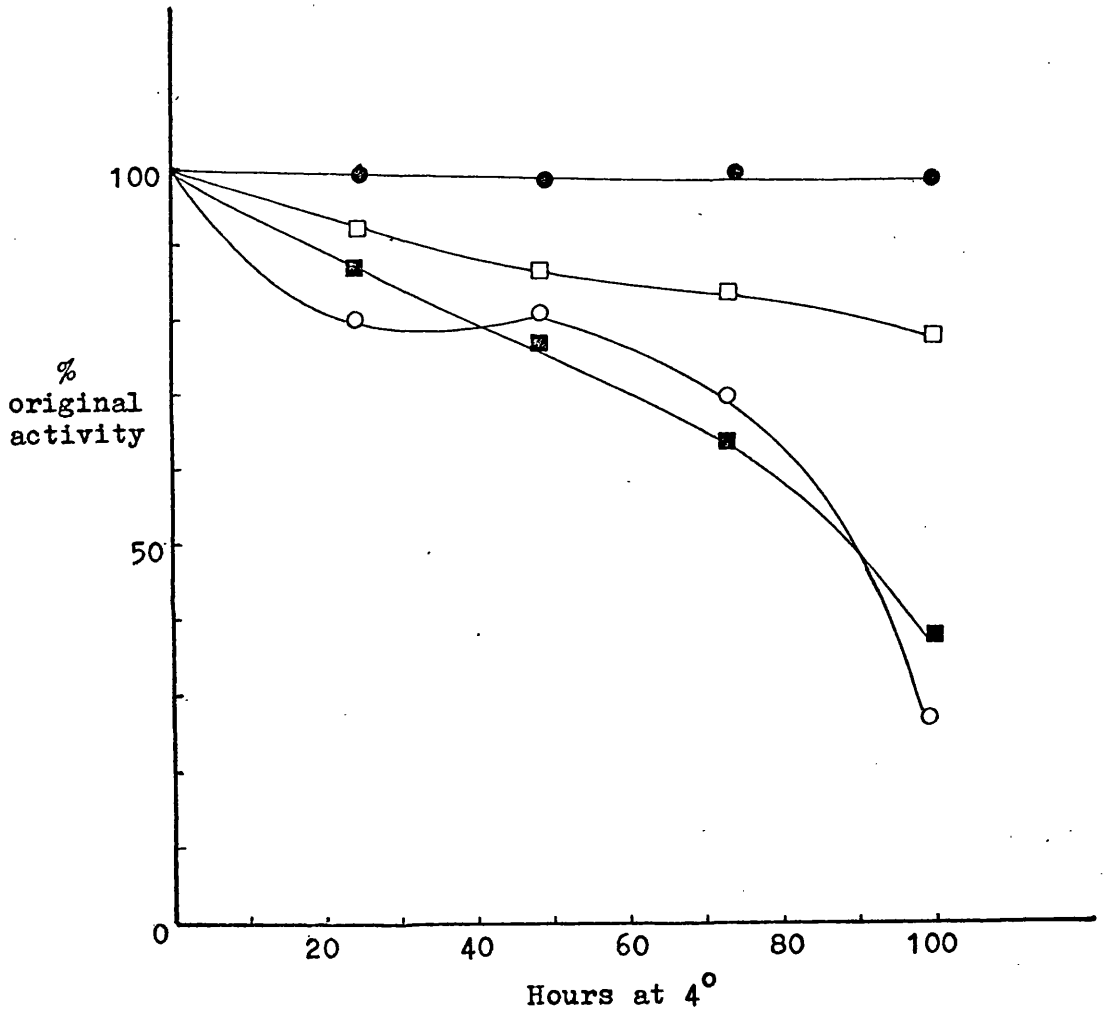
Denaturation of cellulase components of culture filtrate during storage at 4°.

● - β-glucosidase

□ - CMC-ase

■ - Cx-cellulase

○ - Cellulase


100
50
0
% original activity
20
40
60
80
100
Hours at 4°

Storage of Fractions at 4° following Fractionation on Sephadex G200

(Table 5).

Storage Time (Days)	Total units β-glucosidase	Total units CMC-ase 1	Total units CMC-ase 2	Total units Cx-cellulase	Total units cellulase
0	7860	1525	600	750	250
1	7800	1475	480	550	190
2	7750	1490	185	250	nil
3	7800	1450	nil	80	nil
5	7750	1410	nil	nil	nil

fractions which were normally stored at -20° until required were maintained at 4° and samples withdrawn at regular intervals for assay over a period of 5 days. After 2 days no cellulase activity was detected, after 3 days the second CMC-ase activity had disappeared and after 5 days all Cx-cellulase activity was lost, the rates of decay of these activities again being shown to be similar (table 5). During this period no denaturation of β-glucosidase and only slight loss of the major CMC-ase component was detected.

A further attempt was however made to separate the multiple activities of the Cx-cellulase on Sephadex G200 when the aquisition of an upward flow adaptor enabled the outlet of the Pharmacia K15/90 column to be connected into the inlet of the K25/100 column, thereby doubling the effective bed height. No further resolution of the Cx-cellulase component was achieved by this modification. While Sephadex G200 was subsequently used routinely for the separation of β-glucosidase and CMC-ase, fractionation techniques involving gel electrophoresis and ion exchange chromatography were employed in an effort to resolve the uni- or multi-enzymic identity of the third enzymic component for which the term "Cx-cellulase" was retained. Fractions containing this component were freeze dried until required and reconstituted in 0.1 M acetate buffer for ion exchange investigations or in 0.01 M tris-glycine buffer pH 8.3 containing 0.25 M sucrose for gel electrophoresis studies as described in (g) and (h) below.

(g) Ion exchange chromatography

Fractionations of the purified Cx-cellulase on both DEAE- and SE- Sephadex failed to resolve this component which was adsorbed on the anion exchanger at pH 8.0 and eluted at pH 5.5, and adsorbed

Figure 11.

Ion-exchange chromatography.

a) Chromatography of Cx-cellulase on DEAE-Sephadex.

■- Cx-cellulase

○- pH

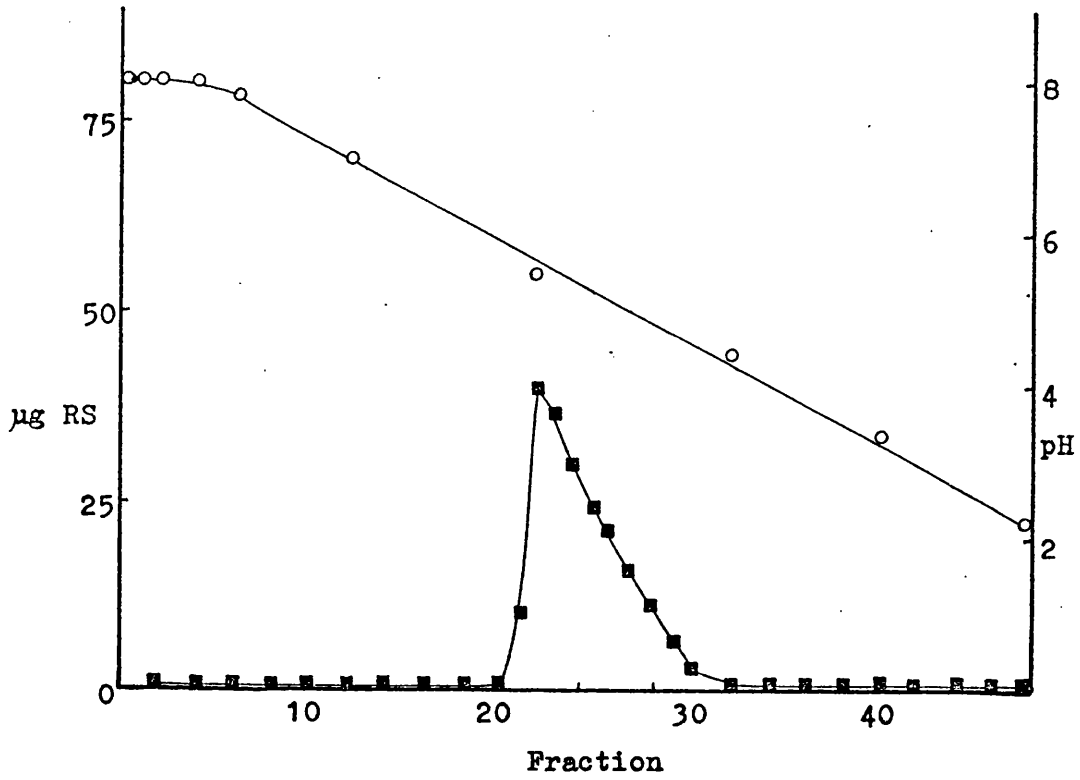
Recovery 83%

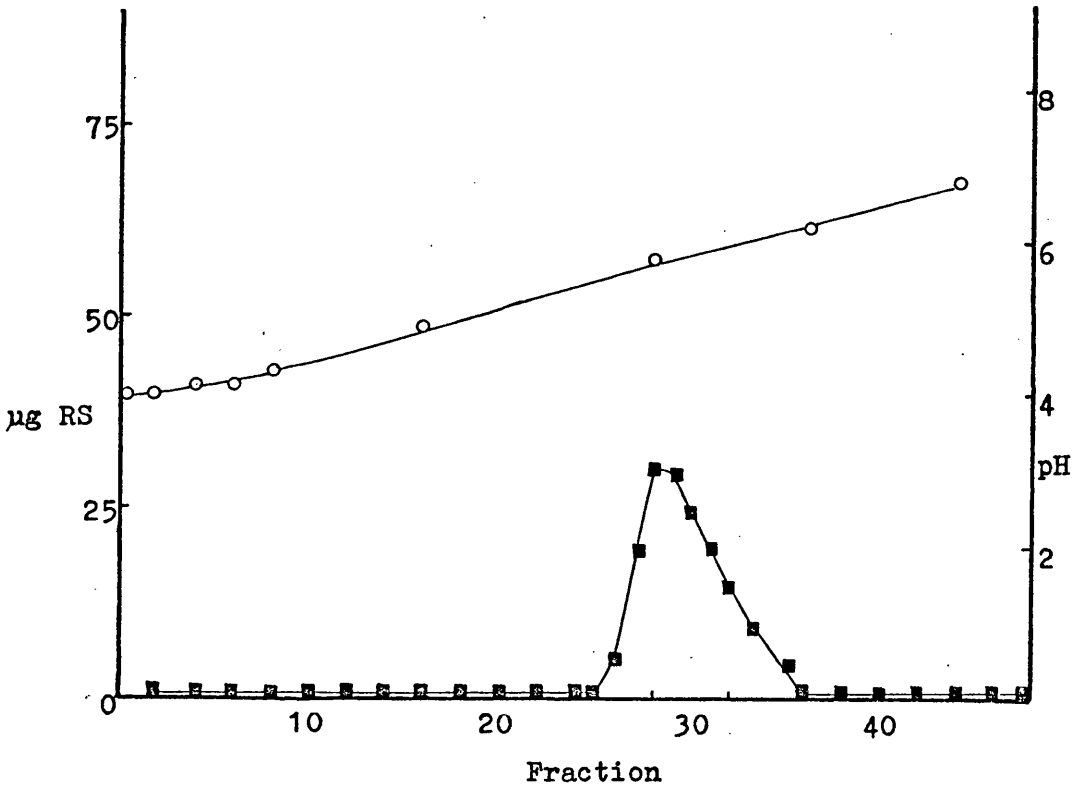
b) Chromatography of Cx-cellulase on SE-Sephadex.

■- Cx-cellulase

○- pH

Recovery 96%


75
50
25
0
µg RS
8
6
4
pH
2
10
20
30
40
Fraction


75
50
25
0
µg RS
8
6
4
pH
2
10
20
30
40
Fraction

on the cation exchanger at pH 4.0, desorption occuring at pH 5.8. In both cases the activity was eluted as a single peak active against CMC, acid swollen cellulose and cotton fibres (fig 11).

(h) Gel electrophoresis

Small scale fractionations of samples of culture filtrate previously purified on Sephadex G75 revealed the presence of a large number of proteins which had remained unresolved during this preparative fractionation. Although the band corresponding to the β-glucosidase component was successfully identified (plate 1), all attempts to locate the remaining cellulolytic enzymes by embedding gels in solidified substrate preparations failed. Attempts to section the gels by means of a refrigerated microtome also proved unsuccessful due to the elastic nature of the polymerized acrylamide.

Despite the high degree of resolution obtained by small scale electrophoresis, the separation of enzymic components by the preparative column proved disappointing (fig 12). This was however believed to be due to an excessive elution chamber volume which was incapable of maintaining the separation of protein bands achieved by electrophoresis. Despite the poor separation, the elution patterns obtained by preparative disc electrophoresis indicated the location of the CMC-ase and Cx-cellulase components in the small stained gels to be the two protein bands possessing slightly greater mobilities than the β-glucosidase component. Rf values based on the bromophenol blue marker were calculated as 29, 37 and 41 for the β-glucosidase, CMC-ase and Cx-cellulase components respectively. As previously all cellulase activity was confined to those fractions containing the Cx-cellulase component, and similarly samples of the fractions containing the

Figure 12.

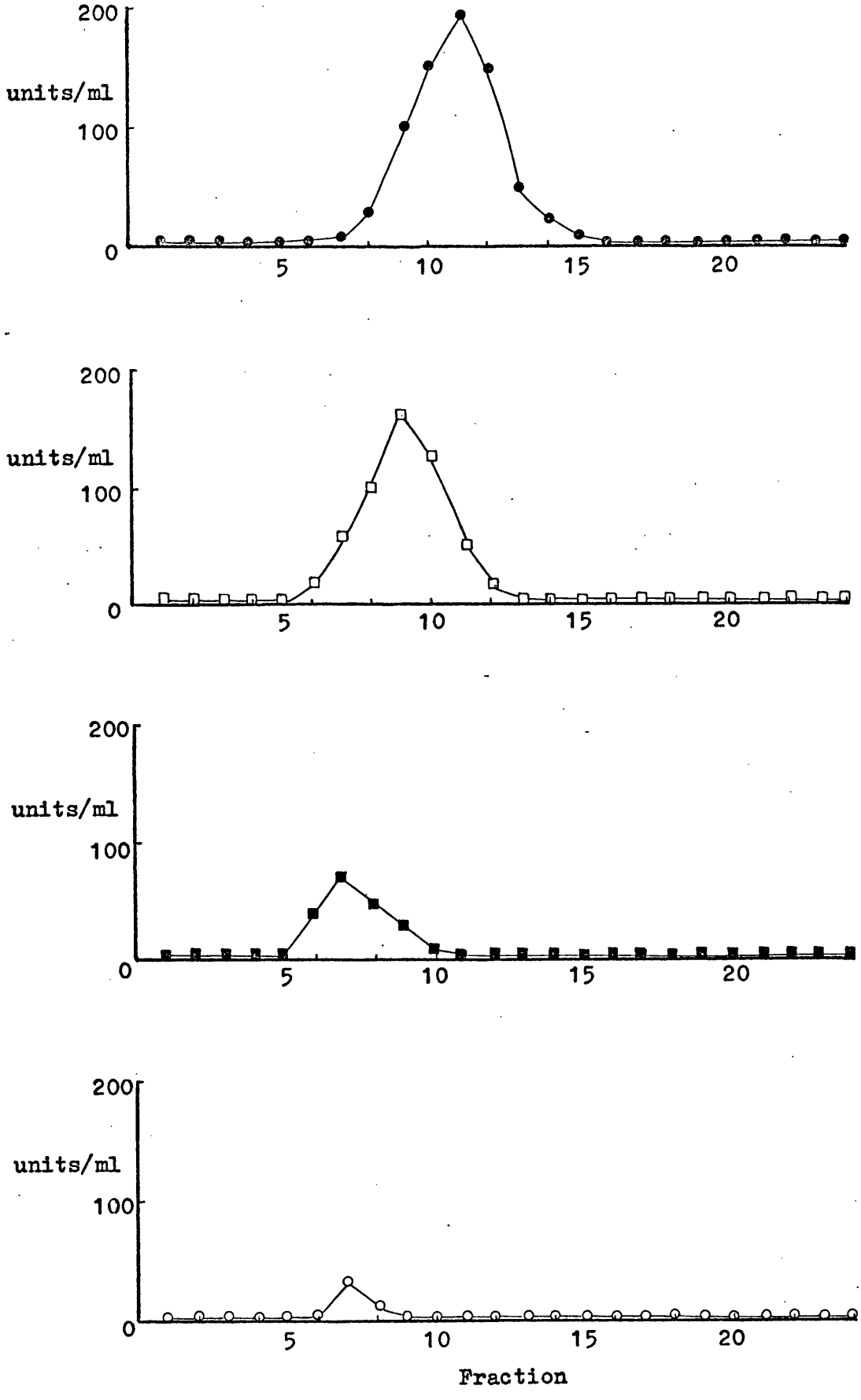
Preparative disc electrophoresis of culture filtrate.

● - β-glucosidase

□ - CMC-ase

■ - Cx-cellulase

○ - Cellulase


200
units/ml
100
0
5
10
15
20
200
units/ml
100
0
5
10
15
20
200
units/ml
100
0
5
10
15
20
200
units/ml
100
0
5
10
15
20
Fraction

low molecular weight CMC-ase, Cx-cellulase and cellulase activities obtained by fractionation on Sephadex G200 were subsequently shown to be recovered as a single peak after being subjected to preparative electrophoresis. After concentration by freeze drying this peak was found to migrate as a single protein band with an Rf of 40 on small scale disc electrophoresis gels.

The application of these established fractionation techniques to the solubilized cell-bound extracts enabled a direct comparison of these components to be made with those found in culture filtrates. The results demonstrated fractionation patterns of extracts from both sources to be identical on Sephadex G200, and the isolated components of the solubilized preparations were subsequently shown to exhibit the same Rf values on acrylamide gels as the cell-free components.

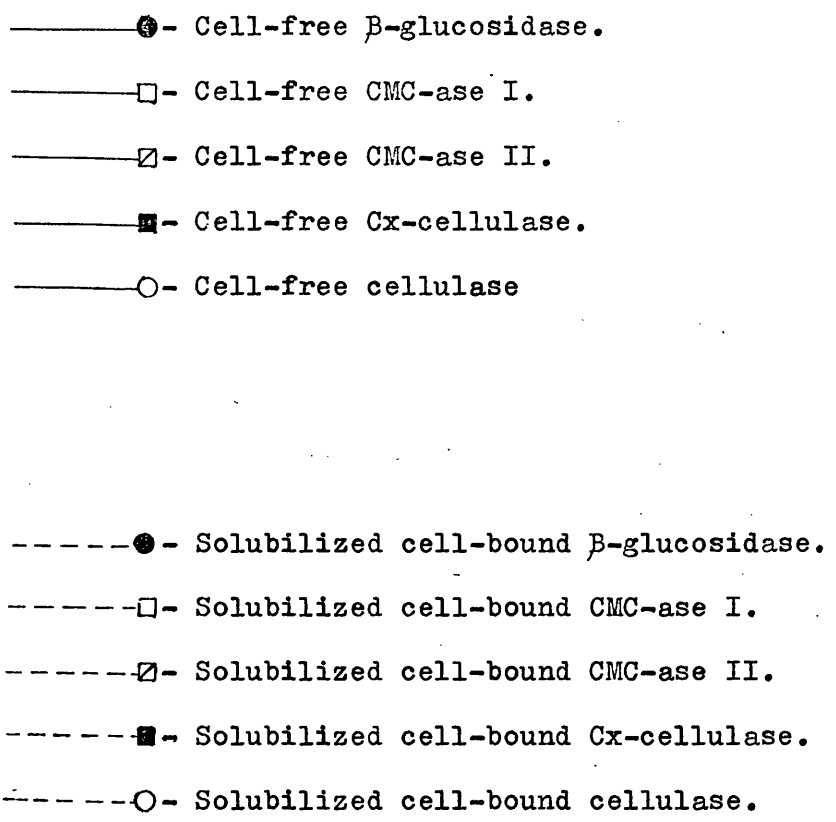
(i) Temperature and pH profiles (figs 13 & 14).

The results of these investigations confirmed that the original choice of pH 5.0 and 40° for routine assays had created incubation conditions which were closely related to the optima required by individual enzymic components. Although these conditions were correct for CMC-ase activity, slight enhancement of β-glucosidase and Cx-cellulase activities were shown to be possible by increasing the pH to 6.0 in both cases and by incubating at 37° for Cx-cellulase and at 45° for β-glucosidase. In view however of the practical difficulties inherent in such modifications at this stage of the investigation particularly in the calculation of enzyme recoveries, and because of the minor increases involved, routine estimations of activity were continued at pH 5.0 and 40°.

Despite differences in relative concentrations, the pH and temperature profiles of both cell-free and solubilized cell-

Figure 13.

Temperature profiles.



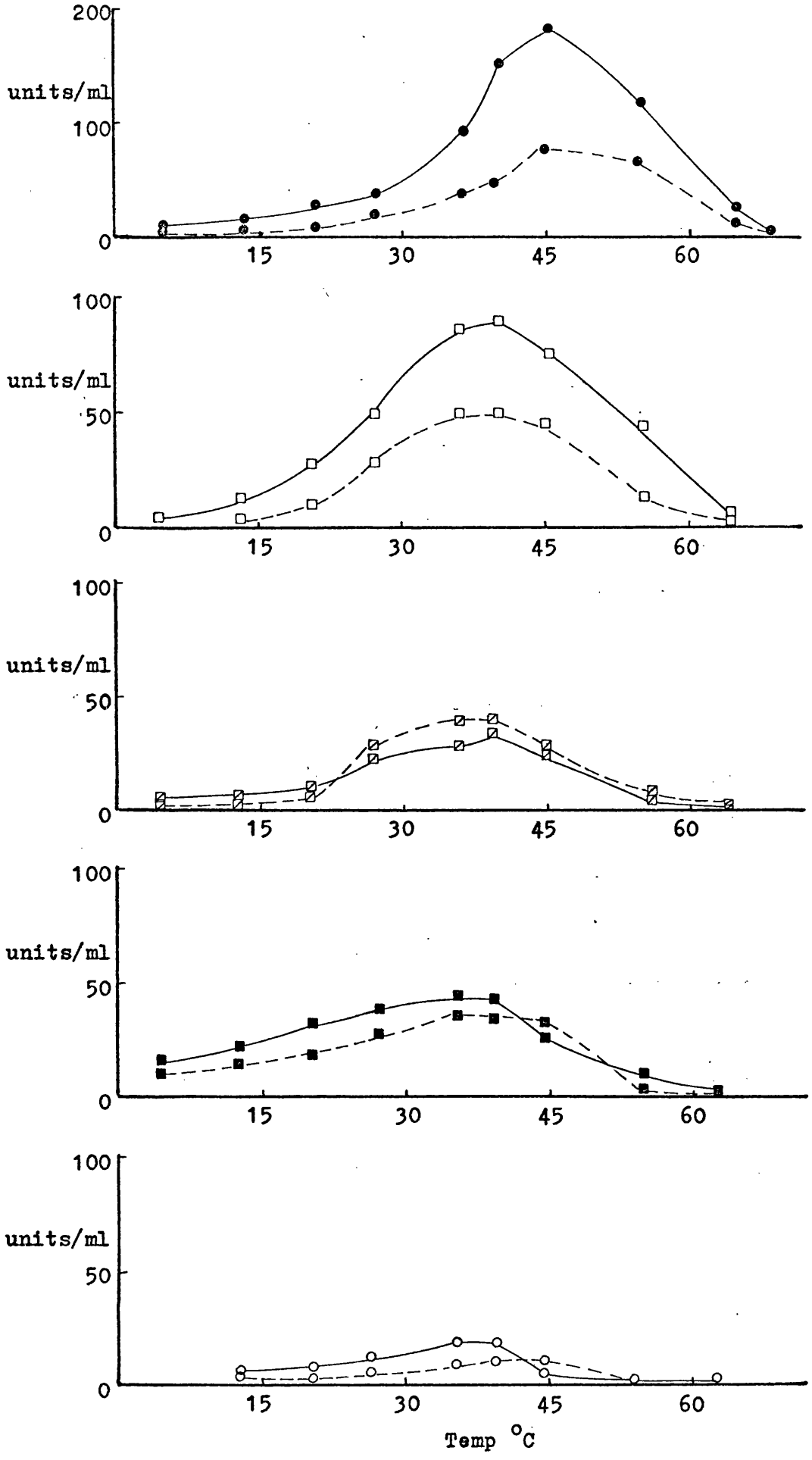
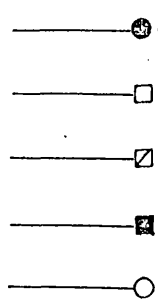
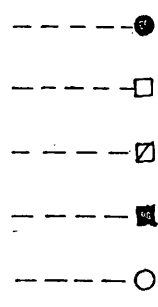
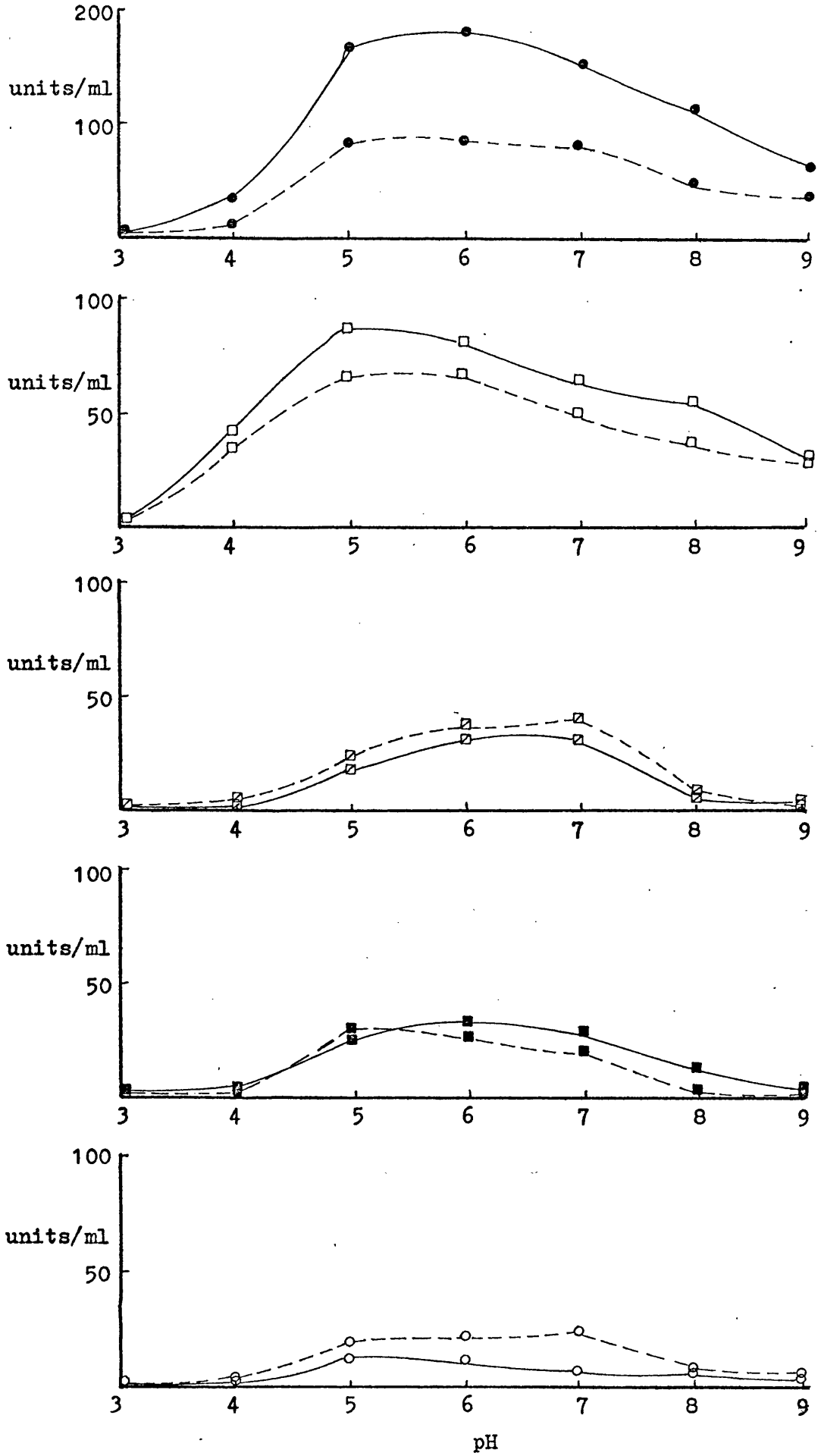

200
units/ml
100
0
15
30
45
60
100
units/ml
50
0
15
30
45
60
100
units/ml
50
0
15
30
45
60
100
units/ml
50
0
15
30
45
60
100
units/ml
50
0
15
30
45
60
Temp °C

Figure 14.

pH profiles.






200
units/ml
100
3
4
5
6
7
8
9
100
units/ml
50
3
4
5
6
7
8
9
100
units/ml
50
3
4
5
6
7
8
9
100
units/ml
50
3
4
5
6
7
8
9
100
units/ml
50
3
4
5
6
7
8
9
pH

bound enzymes were again shown to possess considerable similarities.

(j) Molecular weight determinations

A linear relationship of log MWt against Ve/Vo was established for protein molecules possessing molecular weights between 14,700 and 225,000 when fractionated on Sephadex G200, although the largest protein (catalase) was completely excluded from the gel resulting in a Ve/Vo ratio of 1 (fig 15). The Ve/Vo ratios for β-glucosidase, CMC-ase and Cx-cellulase components were calculated from their elution volumes on this calibrated column as 1.0, 2.18 and 3.18 respectively, indicating a molecular weight of CMC-ase of approximately 45,000 and of Cx-cellulase approximately 11,200. In view of the total exclusion of β-glucosidase, the molecular weight of this component is believed to be in excess of 225,000. While Sephadex G200 is claimed to have a fractionation range of 5,000 to 800,000 for peptides and globular proteins, the molecular weight range of linear dextrans capable of being resolved by this grade of gel is only 1000 to 200,000. These results are thus believed to suggest β-glucosidase to be an extremely large molecule possessing an essentially linear configuration. Although the estimated molecular weights of CMC-ase and Cx-cellulase fall well within the fractionation range of Sephadex G75, no explanation can be given for the lack of resolution of these components on this gel.

(k) Enzyme-substrate affinities (figs 16-23).

Although the desirability of using reasonably pure enzyme preparations for the determination of Michaelis constants is well known, the equal importance of substrate purity is often overlooked, particularly in investigations of enzymes possessing low degrees of specificity. In common with most other depolymerases, cellulolytic

Figure 15.

Molecular weight determination.

Calibration of Sephadex G200 column by fractionation of

1. Lysozyme
2. Pepsin
3. Ovalbumin
4. Serum albumin
5. Catalase

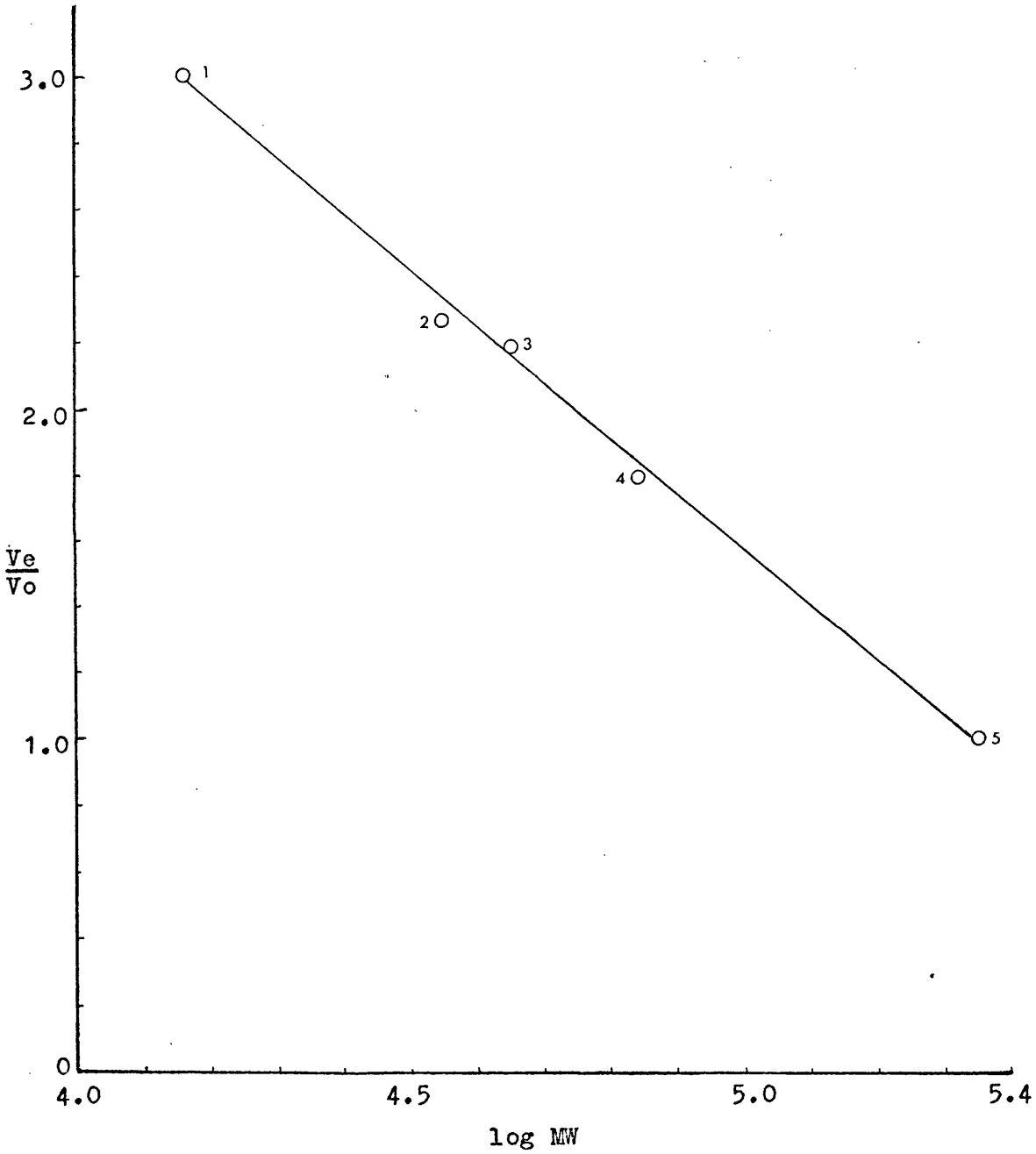

3.0
2.0
1.0
0
Ve
Vo
1
2
3
4
5
4.0
4.5
5.0
5.4
log MW

Figure 16.

Michaelis constant:- β-glucosidase on ONPG

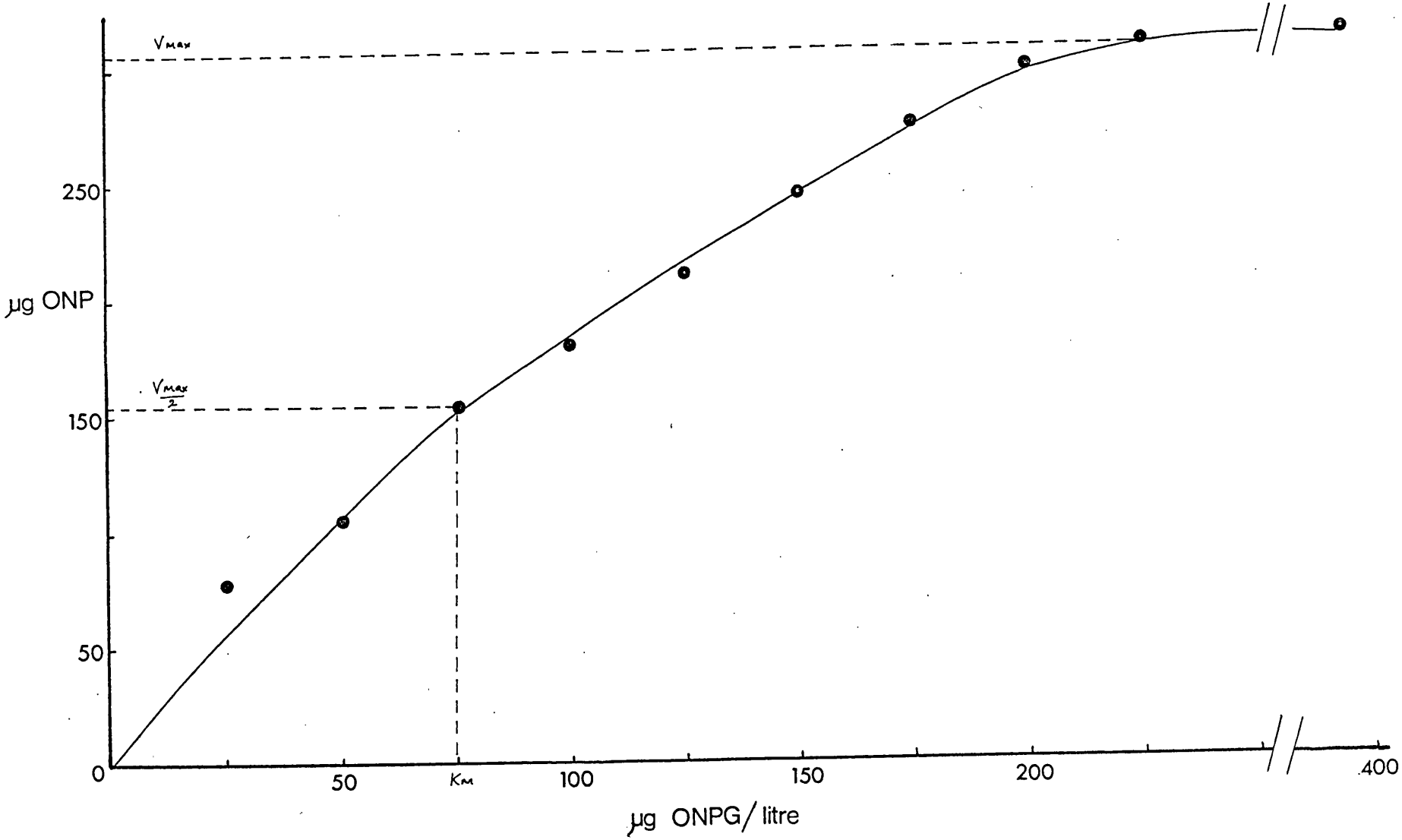
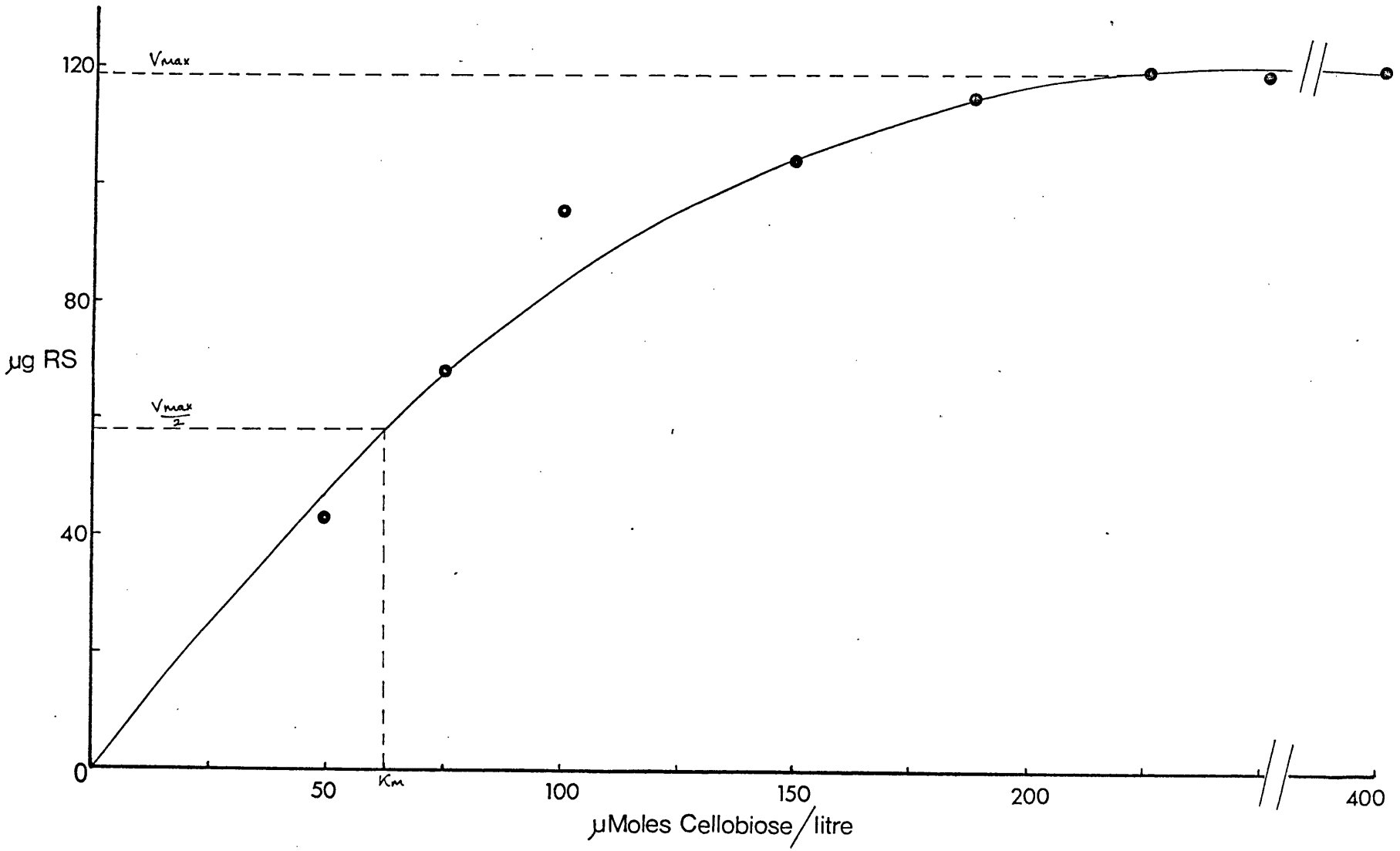

Vmax
250
µg ONP
Vmax/2
150
50
0
50
Km
100
150
200
400
µg ONPG/ litre

Figure 17.

Michaelis constant:- β-glucosidase on cellobiose.


120
Vmax
80
µg RS
Vmax/2
40
0
50
Km
100
150
200
400
µMoles Cellobiose/litre

enzymes are normally active on extremely heterogeneous substrates, the molecules of cellulose and cellulose derivatives exhibiting different susceptibilities to attack according to their degree of polymerization, degree of substitution and architectural features. The situation may be further complicated by a tendency for different enzymic components acting on structurally similar substrates to lack the rigid specificity normally claimed to be an essential feature of enzyme reactions, necessitating more rigid purification of extracts than usually considered adequate. With the exception of β-glucosidase acting on ONPG or cellobiose, calculation of Michaelis constants for the cellulolytic components of C.acremonium were consequently impracticable. In an effort however to obtain some information on the relative affinities of these components for a variety of cellulosic substrates, the increases in activity of constant amounts of enzyme produced by increasing substrate concentrations were plotted (figs 18-23).

The rapid rates of hydrolysis of ONPG and cellobiose noted during earlier investigations were confirmed by the considerable affinity of β-glucosidase for both the natural and artificial substituted substrates. The choice of ONPG in place of cellobiose for routine estimations of this enzyme was based initially on the simplified assay procedure involved, and while the relative affinity of the enzyme for this unnatural substrate may be questioned, the results obtained indicate close similarities in the concentrations of ONPG and cellobiose required to produce 50% saturation of identical samples. Thus the Km for β-glucosidase was calculated as 7.5×10^{-5} molar ONPG and 6.25×10^{-5} molar cellobiose, demonstrating that although the enzyme had a slightly greater affinity for cellobiose, the routine use of ONPG had

Figure 18.

Affinity of β-glucosidase for cellotriose.

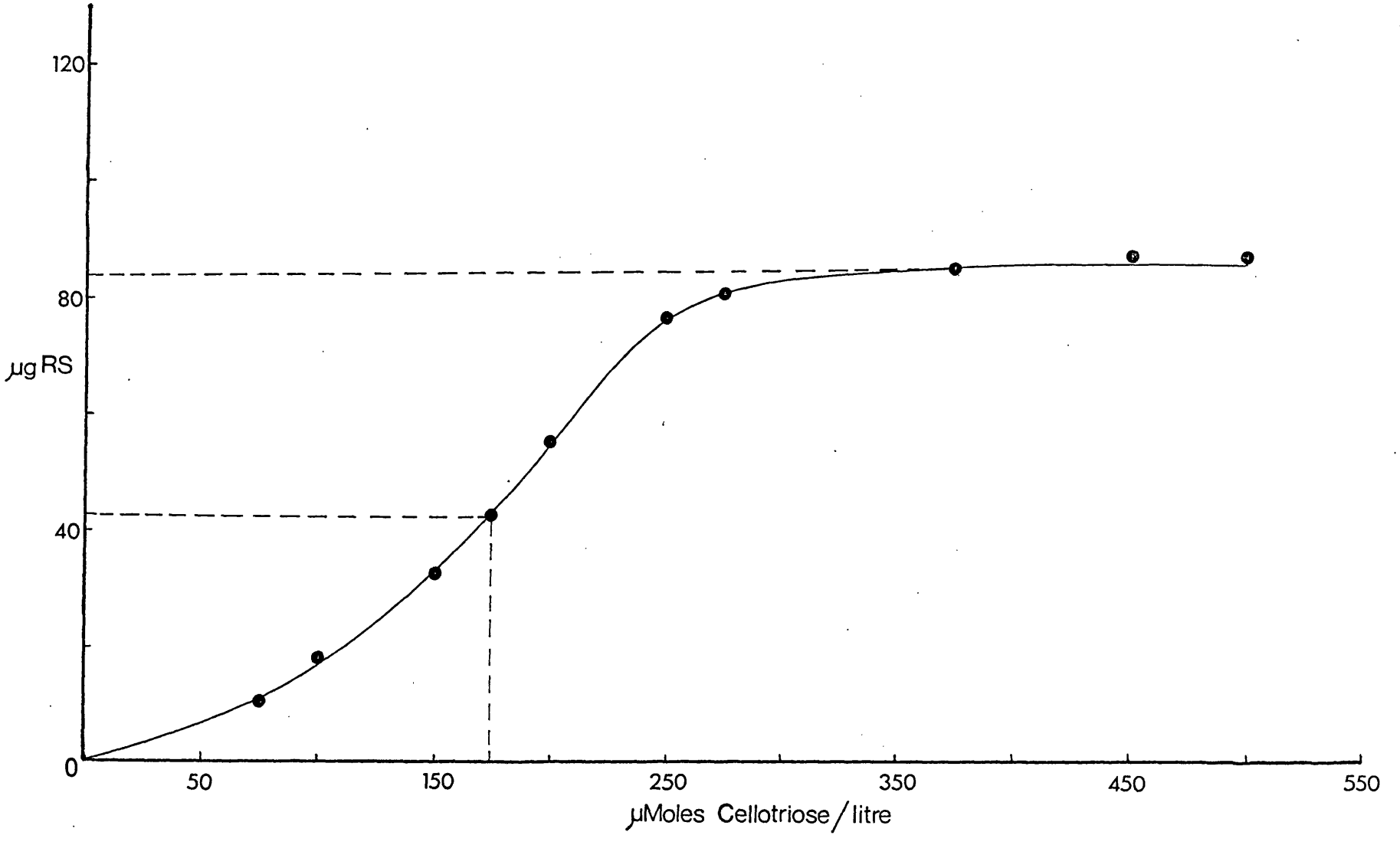

120
80
40
0
µg RS
50
150
250
350
450
550
µMoles Cellotriose / litre

Figure 19.

Affinity of β-glucosidase for cellotetraose.

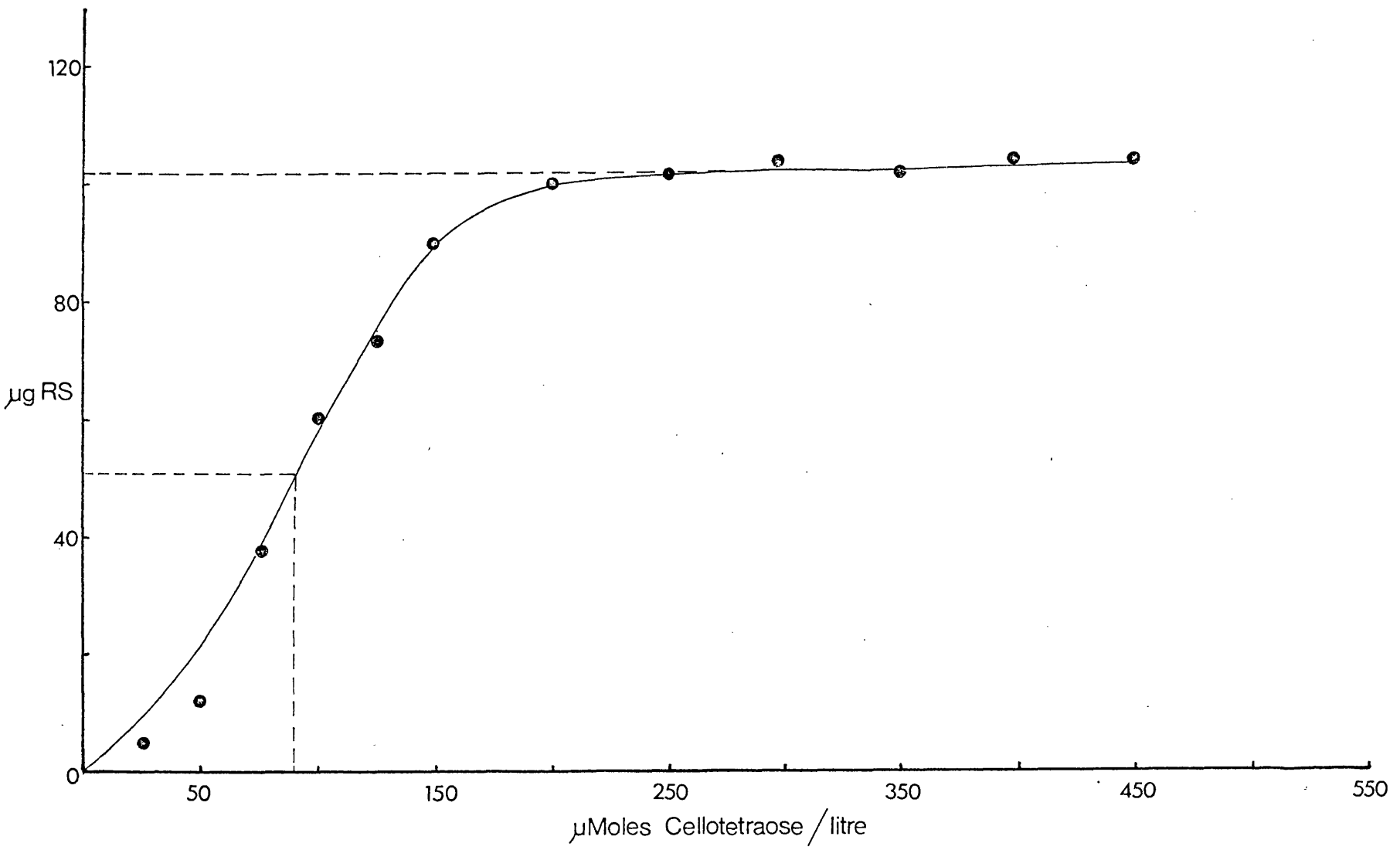

120
80
40
0
µg RS
50
150
250
350
450
550
µMoles Cellotetraose / litre

Figure 20.

Affinity of CMC-ase for soluble fraction of CMC.

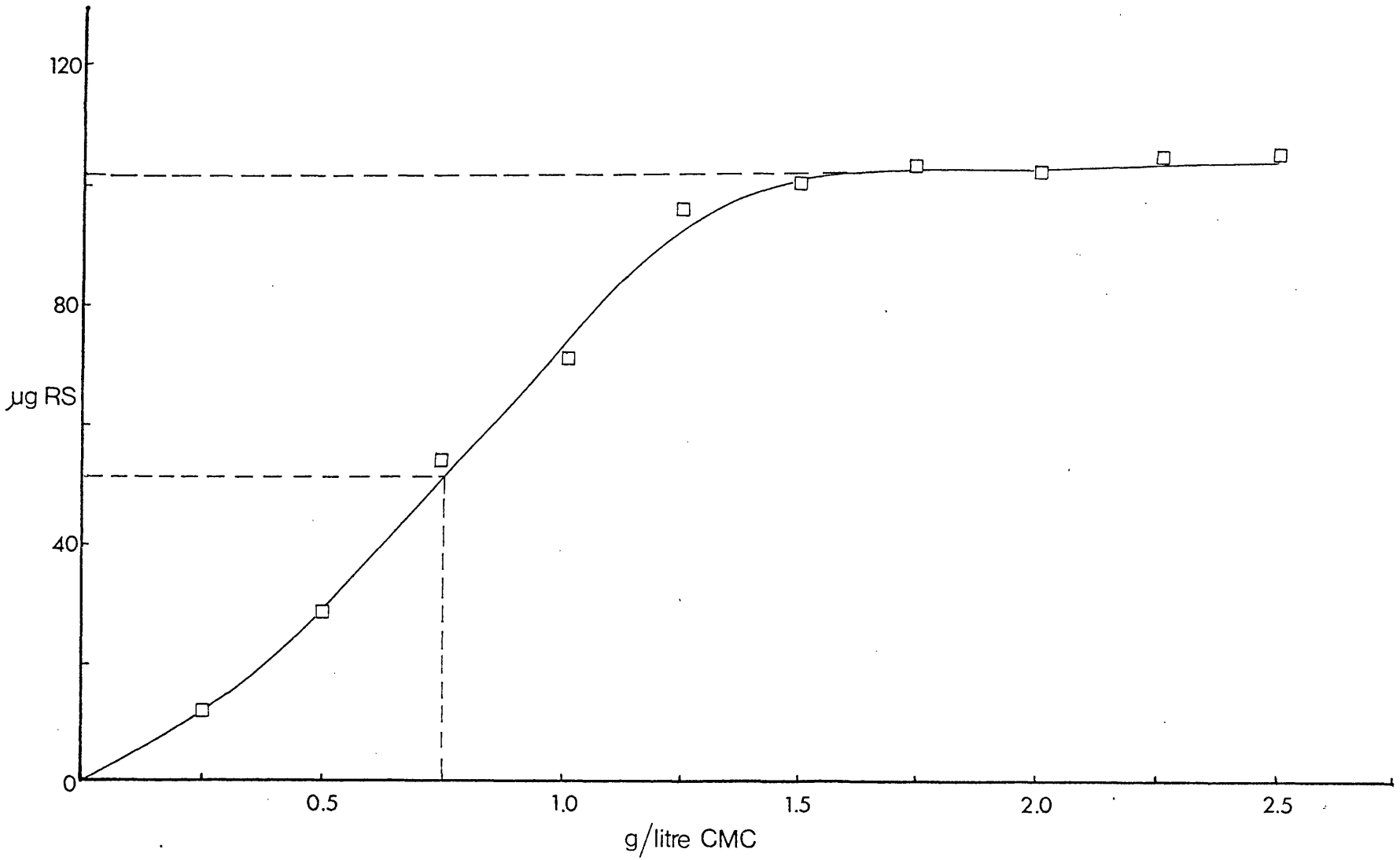

120
80
40
0
µg RS
0.5
1.0
1.5
2.0
2.5
g/litre CMC

Figure 21.

Affinity of Cx-cellulase for soluble fraction of CMC.

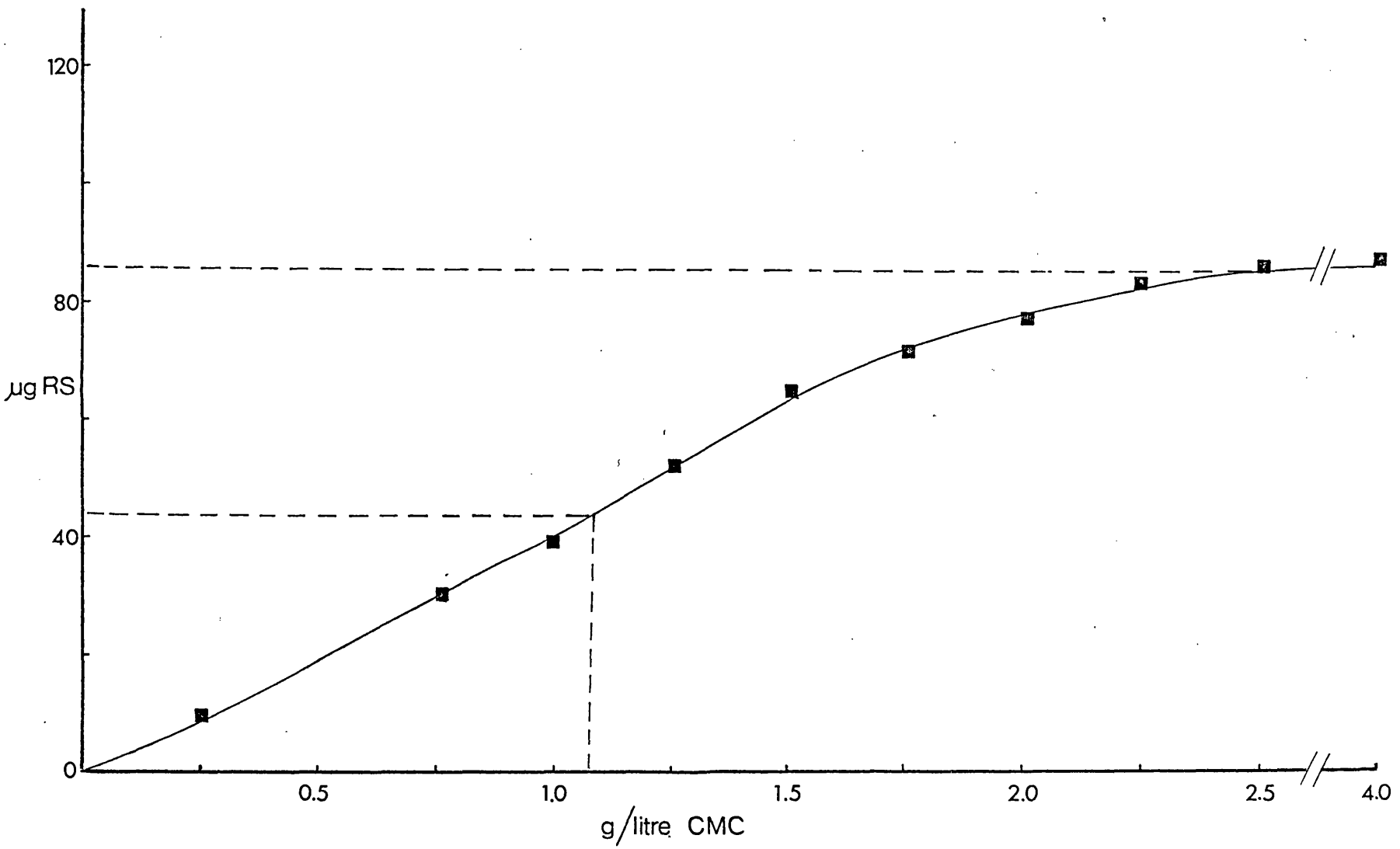

120
80
40
0
µg RS
0.5
1.0
1.5
2.0
2.5
4.0
g/litre CMC

Figure 22.

Affinity of Cx-cellulase for acid swollen ball-milled cellulose.

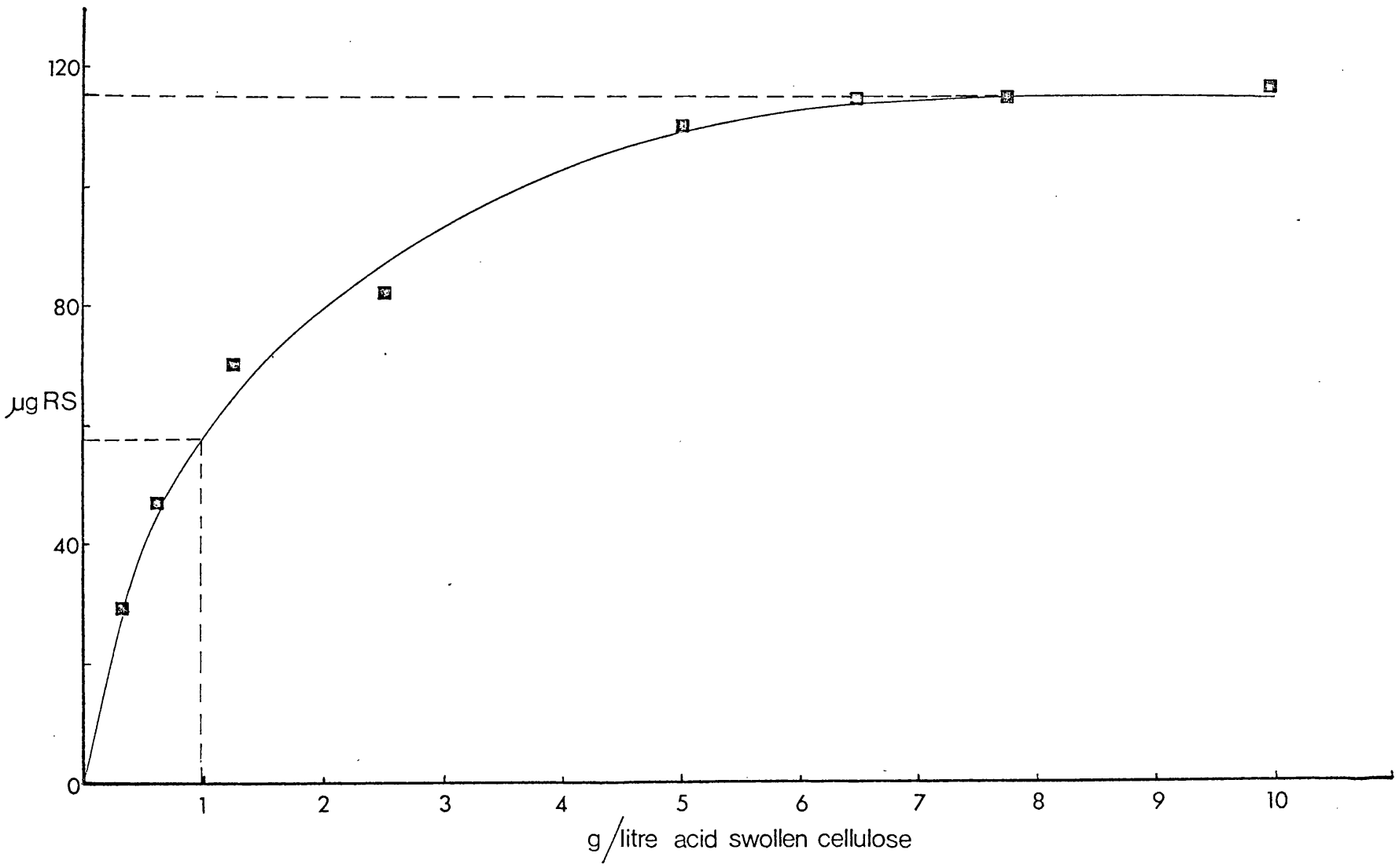
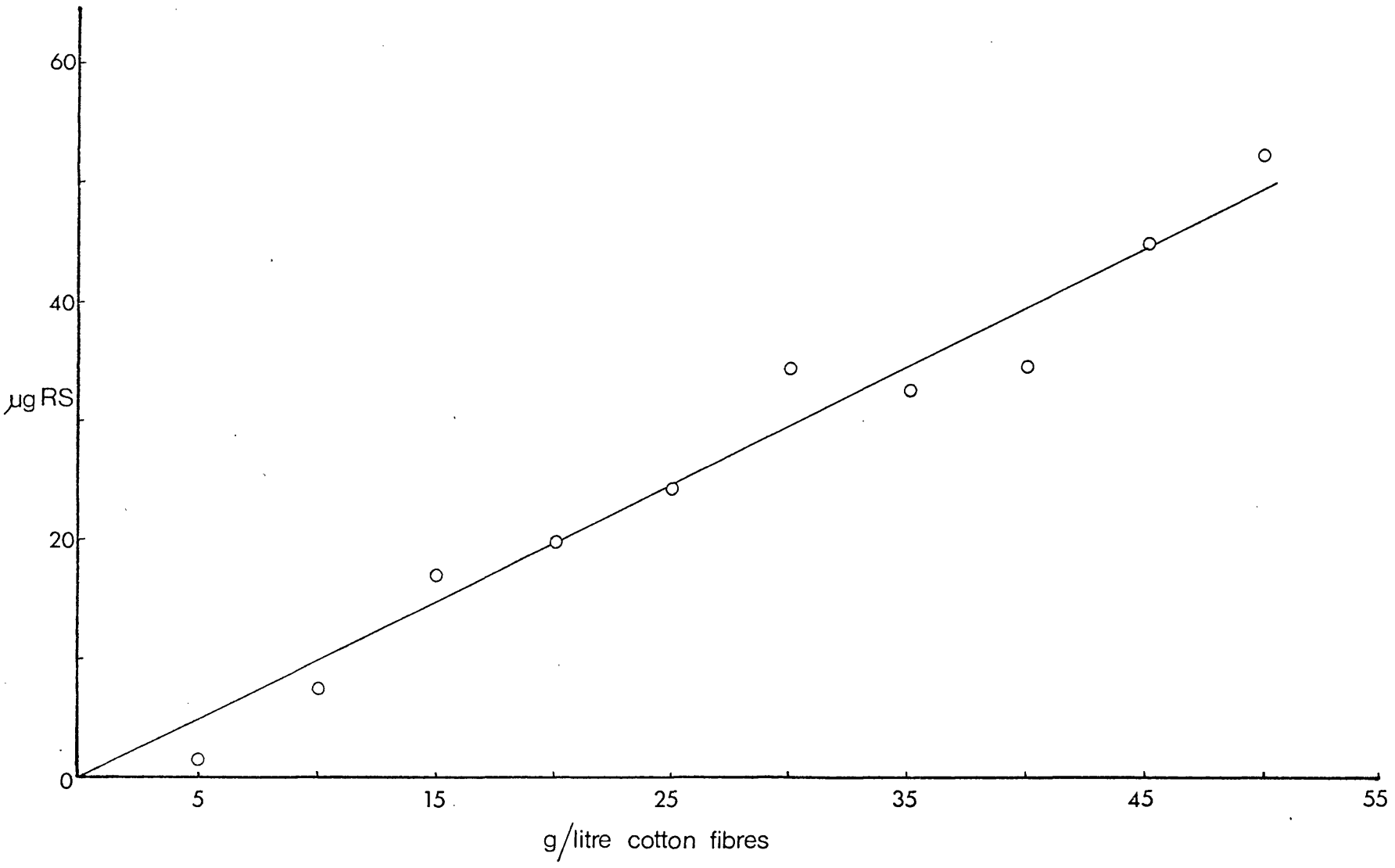

120
80
40
0
µg RS
1
2
3
4
5
6
7
8
9
10
g/litre acid swollen cellulose

Figure 23.

Affinity of Cx-cellulase for cotton fibres.

Each plot is average of three determinations.


60
40
20
0
µg RS
5
15
25
35
45
55
g/litre cotton fibres

resulted in accurate determinations of true cellobiase activity. Although the affinity of β-glucosidase for cellotriose and cellotetraose was also investigated by this technique, estimations of activity by reducing sugar analyses proved unsuitable in the case of these substrates due to the liberation of cellobiose on which the enzyme was considerably more active. The apparently greater affinity of β-glucosidase for cellotetraose than for cellotriose may consequently be due to the release of two molecules of cellobiose from each cellotetraose molecule as opposed to one in the case of cellotriose. Both substrates were however rapidly hydrolysed by β-glucosidase, concentrations of cellotriose and cellotetraose producing 50% saturation being calculated as 17.5×10^{-5} molar and 9.0×10^{-5} molar respectively.

Similar investigations of CMC-ase and Cx-cellulase activities demonstrated a considerable decrease in affinities as the structural complexity of the substrates increased, until the situation was reached where assayable quantities of Cx-cellulase could not be saturated with cotton fibres before adsorption of the liquid phase of the incubation mixtures occured. These results are believed to indicate a limited number of areas present in native cotton fibres which are accessible or susceptible to attack. As noted previously the lack of saturation conditions in incubation mixtures used routinely for cellulase assays was believed to be partly responsible for the low activities measured. In contrast to this general rule, the affinity of Cx-cellulase for the insoluble acid swollen cellulose substrate was found to be similar to that for CMC despite the vastly increased accessibility of the soluble derivative.

No significant variations of affinities for any of the

Figure 24.

Viscometric studies of isolated enzymes.

a) β-glucosidase

● - Autoclaved control

○ - Inhibited by gluconolactone

□ - Untreated

b) CMC-ase

● - Autoclaved control

○ - Inhibited by gluconolactone

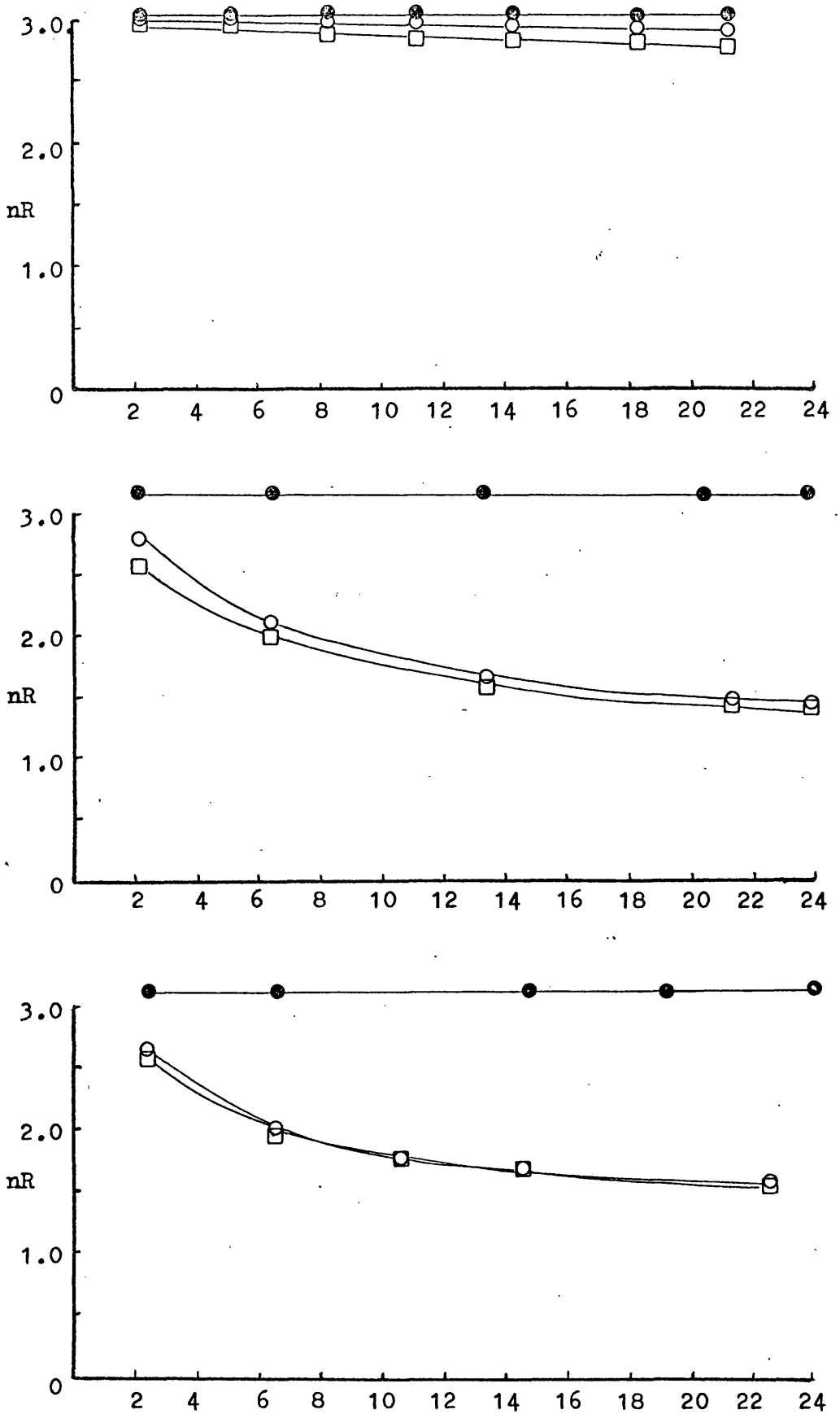
□ - Untreated

c) Cx-cellulase

● - Autoclaved control

○ - Inhibited by gluconolactone

□ - Untreated


3.0
2.0
nR
1.0
0
2 4 6 8 10 12 14 16 18 20 22 24
3.0
2.0
nR
1.0
0
2 4 6 8 10 12 14 16 18 20 22 24
3.0
2.0
nR
1.0
0
2 4 6 8 10 12 14 16 18 20 22 24
min.

substrates investigated could be shown to exist between enzyme components isolated from culture filtrates or solubilized from cell debris.

(l) Viscometric studies of isolated enzymes

Further information on the mode of attack of cellulose substrates was obtained as a result of viscometric studies using isolated enzymic components (fig 24). While characteristically endoenzymic patterns of degradation of CMC were exhibited by CMC-ase and Cx-cellulase, the β-glucosidase component was found to be responsible for a slight linear decrease in viscosity. Although the concentration of β-glucosidase was similar to that normally present in culture filtrates, the extent of this hydrolysis was less than that measured in crude filtrates heated for 10 min at 100°. Similar heat treatment of the isolated β-glucosidase component resulted in the destruction of activity of this purified enzyme, and the addition of gluconolactone produced almost complete inhibition. This was in contrast to the activity of heat treated filtrates which was reduced but not completely inhibited by gluconolactone, demonstrating that this thermostable activity could not be explained solely in terms of residual β-glucosidase. The lack of inhibitory action of gluconolactone on both the CMC-ase and Cx-cellulase components indicated that traces of either of these enzymes could contribute to the residual activity.

(m) S-factor activity and tensile strength determinations

Although the Cx-cellulase component was found to be responsible for most of the S-factor activity and loss of tensile strength of cotton fibres produced by cell-free extracts, a slight increase in the uptake of alkali and a detectable reduction of strength was also noted in fibres previously exposed to the CMC-ase component.

S-Factor Activity (Table 6).

Treatment	Weight of cotton before treatment (mg)	Average (of 3) weight after treatment (mg)
Control	20	64
β-glucosidase (cell-free, 300 u/ml)	20	63
β-glucosidase (cell-bound, 300 u/ml)	20	64
CMC-ase (cell-free, 250 u/ml)	20	68
CMC-ase (cell-bound, 250 u/ml)	20	66
Cx-cellulase (cell-free, 150 u/ml)	20	76
Cx-cellulase (cell-bound, 150 u/ml)	20	77
Fresh culture filtrate	20	82

Tensile Strength Determinations (Table 7).

Treatment	Average (of 5) breaking strength(Kg)	% Reduction
Control	0.65	-
β-glucosidase (cell-free, 300 u/ml)	0.64	2.0%
β-glucosidase (cell-bound, 300 u/ml)	0.65	nil
CMC-ase (cell-free, 250 u/ml)	0.53	19.0%
CMC-ase (cell-bound, 250 u/ml)	0.50	23.0%
Cx-cellulase (cell-free, 150 u/ml)	0.32	51.0%
Cx-cellulase (cell-bound, 150 u/ml)	0.34	48.0%
Fresh culture filtrate	0.29	56.0%

Similar investigations involving solubilized cell-bound enzymes at equivalent concentrations demonstrated no apparent variation from the results obtained with cell-free extracts. (tables 6 & 7).

(n) X-ray diffraction studies (plate 6).

X-ray diffraction patterns of the four types of insoluble cellulosic substrates used in this investigation (cotton wool, acid swollen/ball milled cellulose, dried and undried cotton boll fibres) were initially believed to indicate considerable variations in crystalline structure which could account for the inability of C.acremonium cultures to completely degrade dried native cotton fibres while being capable of rapidly solubilizing undried boll fibres and acid swollen cellulose. As a high degree of crystallinity is generally indicated in Debye-Scherrer powder patterns by sharp resolution of component lines, the diffused halos observed in patterns produced by the latter easily degraded substrates were believed to indicate the presence of considerable amounts of amorphous cellulose. Similarly the marginally finer resolution of lines in patterns produced by residual short fibres was thought to suggest a slightly greater degree of crystallinity than that of the original fibrous cotton. It should be noted here that some loss of definition has occurred during the reproduction of these patterns in this report.

Further investigations revealed however that considerable variations of line resolution and density could be produced by differences in the extent to which the samples were compressed during packing. In addition the suggestion made by Preston (1952) that water halos are capable of masking cellulose reflections was also investigated in view of the fact that both the acid swollen cellulose and undried boll fibres had been examined in

Plate 6.

X-ray diffraction patterns.

a) Acid swollen ball-milled cellulose (aqueous).

b) Undried cotton boll fibres (aqueous).

c) Non-absorbent cotton wool (dry).

d) Residual short cotton fibres (dry).

aqueous preparations. X-ray diffraction patterns produced by Lindemann tubes containing distilled water confirmed that the diffused halos observed in patterns of these substrates were in fact water halos, and not due to amorphous regions.

(o) Refractive index studies (table 8).

Changes in refractive index which accompany modifications of crystal lattice, orientation or chemical nature of cellulose molecules have been claimed to be observable by immersion methods (Rollins & Tripp, 1963). A colourless solid immersed in a liquid of identical refractive index becomes invisible, and under these conditions a small change in refractive indices produces a bright (Becke) line at the solid/liquid interface. When studied microscopically, the Becke line moves towards the medium of higher refractive index when the focus is raised and vice-versa. In view of the fact that these workers observed a fall in refractive index when native cotton fibres were mercerized, and noted a similar decrease as the degree of substitution of modified celluloses increased (both of these treatments involving a decrease in crystallinity), it was hoped that this immersion method would provide some information on the relative degrees of crystallinity of native cotton and short residual fibres. The slightly higher degree of crystallinity of the residual fibres indicated by the results of this investigation was supported by the additional observation that while considerable variations in refractive index were noted at various points along a single cotton fibre, this was found to be less apparent in the case of the residual short fibres, indicating a more homogeneous crystalline structure.

Refractive Index Studies (Table 8).

Refractive Index	Residue		Cotton Fibres	
	Lines moving into fibre	Lines moving out of fibre	Lines moving into fibre	Lines moving out of fibre
1.56404	7	18	0	25
1.56325	8	17	0	25
1.56258	8	17	1	24
1.55990	9	16	6	19
1.55884	12	13	7	18
1.55750	12	13	8	17
1.55616	16	9	12	13
1.55427	22	3	21	4
1.55292	23	2	22	3
1.55150	25	0	25	0

(p) Paper chromatography of incubation mixtures (table 9).
Despite the inability of paper chromatography to resolve products of enzymic attack possessing a DP greater than cellotetraose (Rf 7.0), some useful information on the possible modes of action of the enzymic components was obtained as a result of these qualitative studies.

(i) β-glucosidase. In addition to cellobiose, this enzyme actively hydrolysed cellotriose and cellotetraose while exhibiting no detectable action on any of the more complex cellulose substrates.

(ii) CMC-ase. Although completely inactive on cellobiose and cellotriose, CMC-ase action was shown to overlap that of β-glucosidase in the case of cellotetraose. In contrast however to the liberation of all intermediates by β-glucosidase, the hydrolysis of cellotetraose by CMC-ase resulted only in the production of cellobiose, demonstrating the action of the enzyme to be confined to the central bond of the tetramer. The absence of glucose in incubation mixtures containing CMC similarly confirmed the inability of CMC-ase to attack the terminal glycosidic bond at either the reducing or non-reducing chain ends.

(iii) Cx-cellulase. Chromatographic analyses of incubation mixtures revealed the wide range of substrates susceptible to attack by this enzyme to be even more extensive than had initially been suspected, the only substrate not hydrolysed being cellobiose. Although the action of Cx-cellulase on cellotriose and cellotetraose was limited, the pattern of hydrolysis of the tetramer was observed to be significantly different from that produced by CMC-ase in that equal bond susceptibility was

Paper Chromatography of Digest Mixtures. (Table 9).

Enzyme	Substrate	Sugars Identified				
		glucose	cellobiose	cellotriose	cellotetraose	residue on origin
β-glucosidase	cellobiose	+	+ (rs)	-	-	-
"	cellotriose	+	+	+ (rs)	-	-
"	cellotetraose	+	+	+	+ (rs)	-
"	CMC	-	-	-	-	+ (rs)
"	A.S.cellulose	-	-	-	-	-
"	cotton	-	-	-	-	-
CMC-ase	cellobiose	-	-	-	-	-
"	cellotriose	-	-	+ (rs)	-	-
"	cellotetraose	-	+	-	+ (rs)	-
"	CMC	-	+	+	+	+ (rs)
"	A.S.cellulose	-	-	-	-	-
"	cotton	-	-	-	-	-
Cx-cellulase	cellobiose	-	-	-	-	-
"	cellotriose	±	±	+ (rs)	-	-
"	cellotetraose	±	±	±	+ (rs)	-
"	CMC	+	+	+	+	+ (rs)
"	A.S.cellulose	+	+	+	+	+
"	cotton	±	±	±	±	±

(rs denotes residual substrate)

indicated. Similarly, all intermediates including glucose capable of being resolved by paper chromatography were detected during the degradation of CMC, acid swollen cellulose and cotton, although in extremely small quantities in the latter case.

(q) Column chromatography of hydrolysates, (fig.25).

Fractionations of samples of CMC on Sephadex G25 before and after exposure to C.acremonium cellulolytic enzymes revealed almost identical patterns of degradation produced by the CMC-ase and Cx-cellulase components. Both enzymes were responsible for an extensive general reduction of DP, and exhibited no apparent preference for any substrate molecule possessing a specific DP. β-glucosidase produced no detectable change in the molecular weight distribution of this substrate. The mode of hydrolysis of CMC by CMC-ase and Cx-cellulase was noted to be similar to the results obtained by Bourne and Pierce (1970) who investigated the depolymerization of β-glucan by β-glucanase. These workers fractionated samples of incubation mixtures on Biogel P2 and P100, and observed that while the initial molecular weight distribution of the β-glucan was 1-20 x 10^6, this was reduced to a maximum of 100,000 within 3h at 30^o, and to less than 5,000 after 24h.

The degradation of acid swollen cellulose and native cotton fibres by the Cx-cellulase component was characterized by an inability to detect soluble high DP products of attack. These observations suggested that either the main hydrolytic products are short chain oligosaccharides, or that the hydrolysis of any high DP soluble molecules would be considerably more rapid than their rate of release, rendering their detection extremely

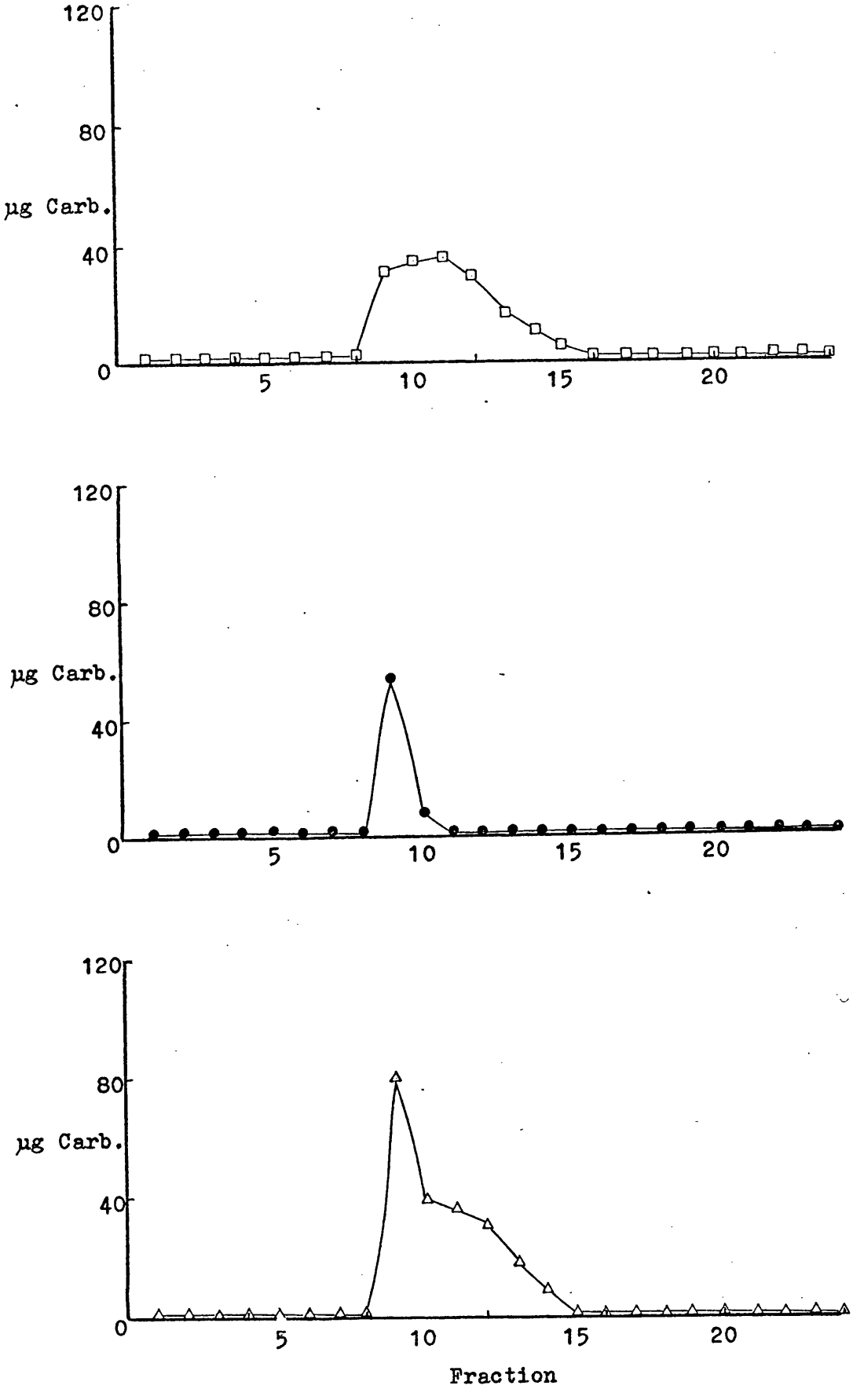
Figure 25.

Column chromatography of hydrolysates on Sephadex G25.

a) □- Soluble fraction of CMC (control).

b) ●- β-glucosidase (control).

c) △- CMC plus β-glucosidase.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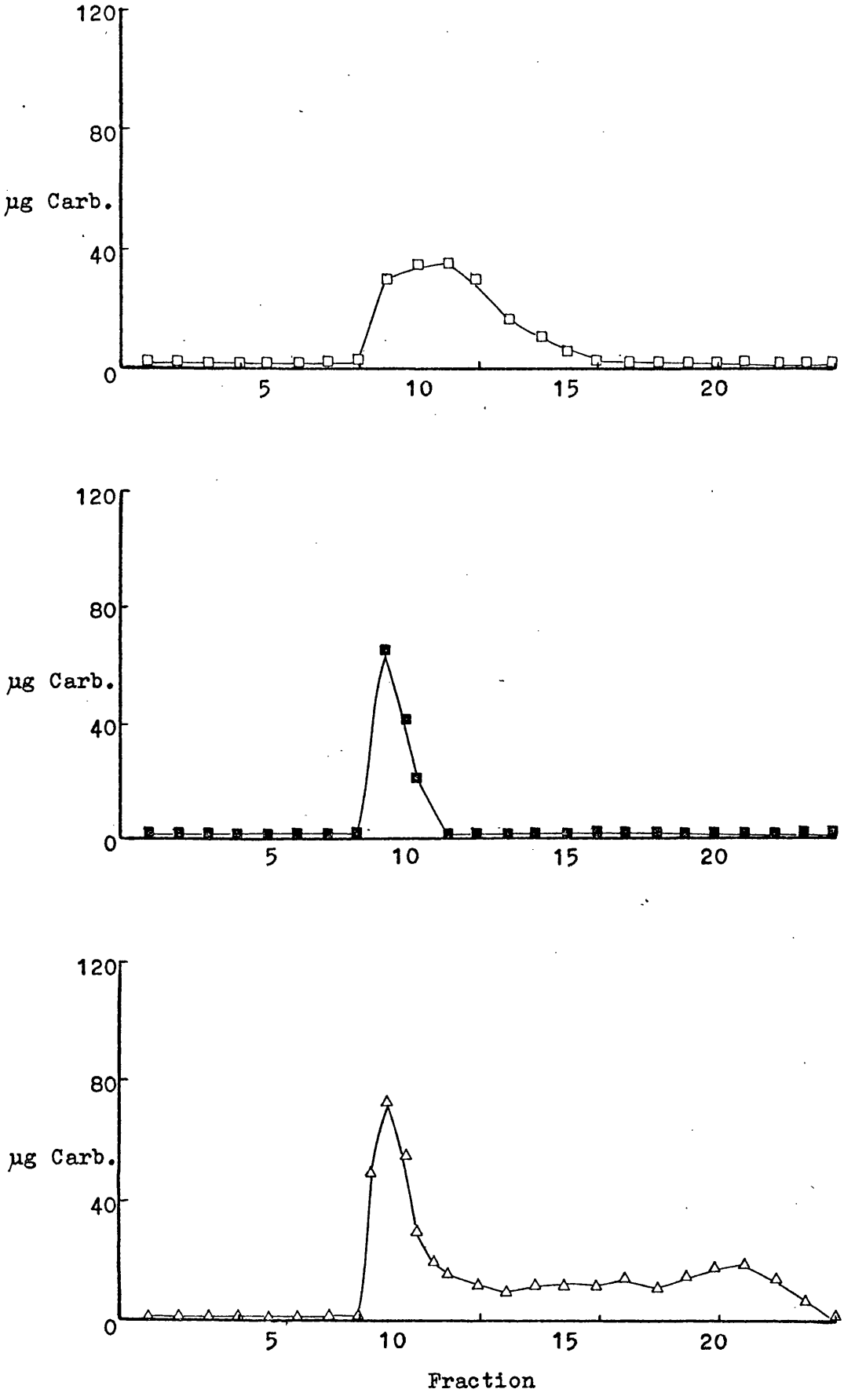
80
µg Carb.
40
0
5
10
15
20
120
80
µg Carb.
40
0
5
10
15
20
120
80
µg Carb.
40
0
5
10
15
20
Fraction

Figure 25 (cont).

d) □- Soluble fraction of CMC (control).

e) ■- CMC-ase (control).

f) △- CMC plus CMC-ase.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80
µg Carb.
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120
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µg Carb.
40
0
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10
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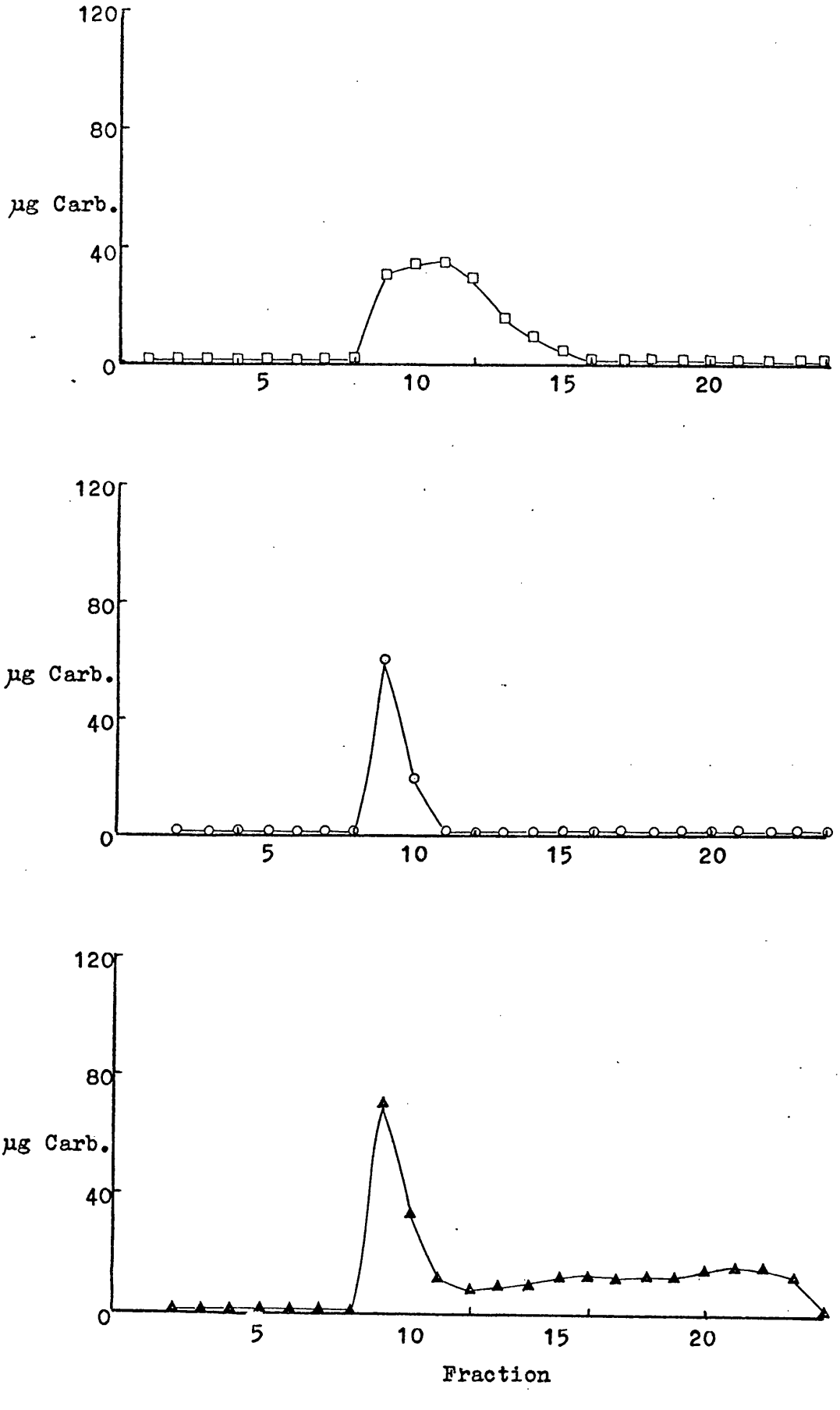
20
120
80
µg Carb.
40
0
5
10
15
20
Fraction

Figure 25 (cont).

g) □- Soluble fraction of CMC (control).

h) ○- Cx-cellulase (control).

i) ▲- CMC plus Cx-cellulase.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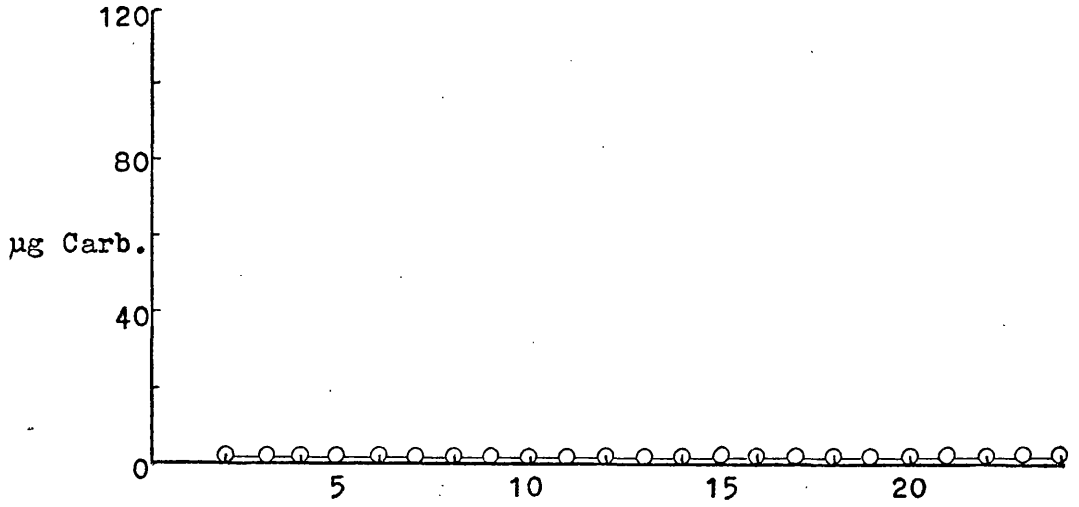
80
µg Carb.
40
0
5
10
15
20
120
80
µg Carb.
40
0
5
10
15
20
120
80
µg Carb.
40
0
5
10
15
20
Fraction

Figure 25 (cont).

j) ○ - Supernatant from centrifuged acid swollen ball-milled cellulose (control).

k) ■ - Cx-cellulase (control).

l) △ - Acid swollen ball-milled cellulose plus Cx-cellulase (supernatant from centrifuged hydrolysate).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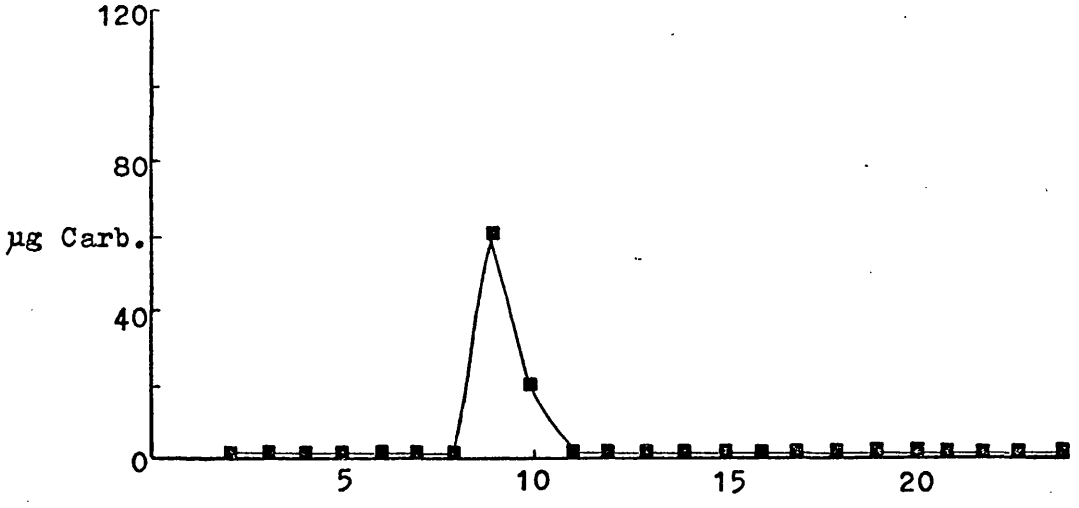
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µg Carb.
40
0
5
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15
20

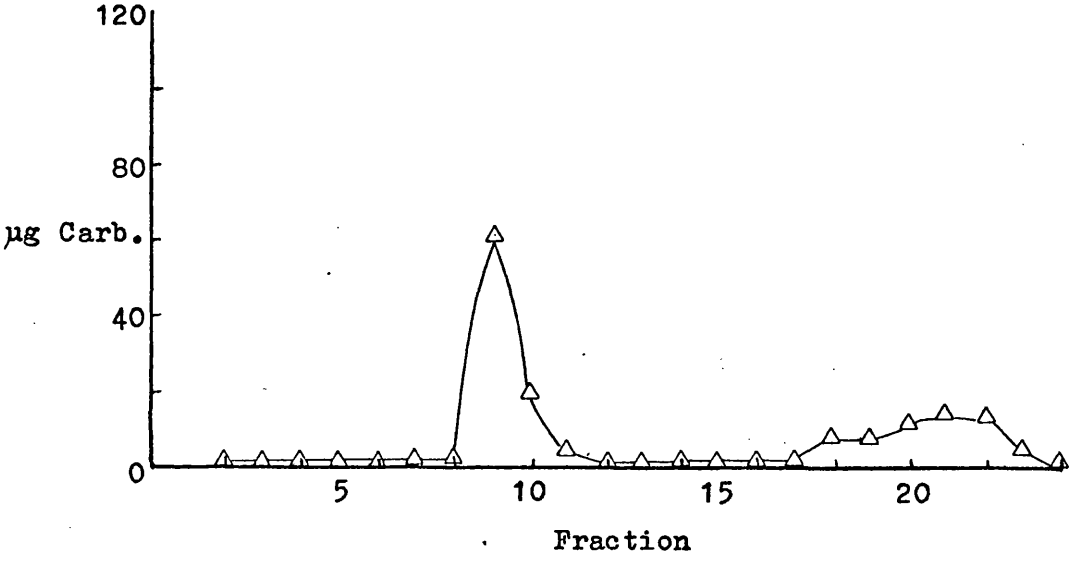
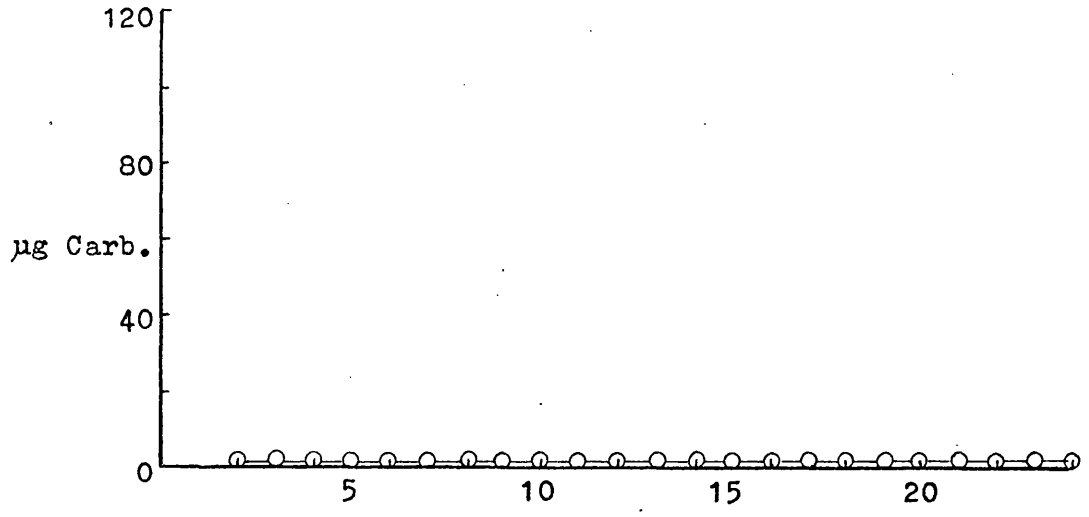

120
80
µg Carb.
40
0
5
10
15
20
Fraction

Figure 25 (cont).

m) ○- Supernatant from centrifuged suspension of cotton fibres (control).

n) □- Cx-cellulase (control).

o) ●- Cotton fibres plus Cx-cellulase (supernatant from centrifuged hydrolysate).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µg Carb.
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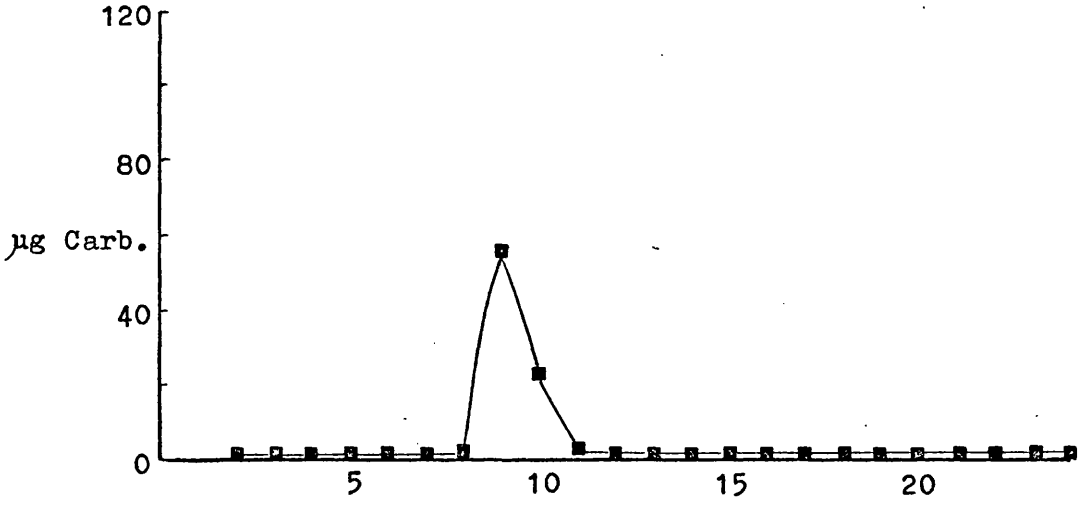
5
10
15
20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µg Carb.
40
0
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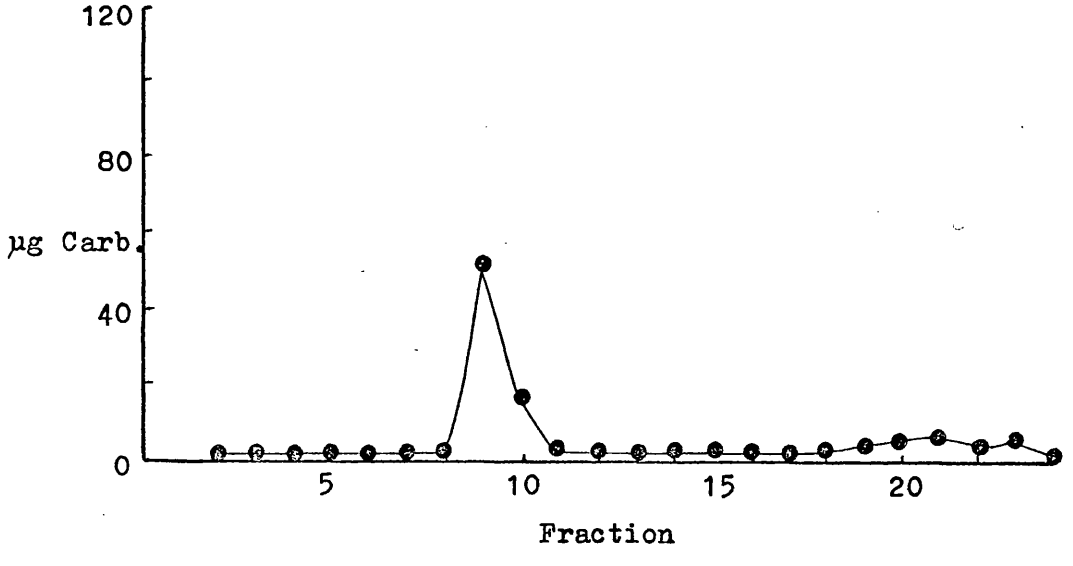
10
15
20


120
80
µg Carb.
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0
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10
15
20
Fraction

difficult.

(r) Specific activities (table 10).

Although the specific activity of an enzyme is usually defined as the activity per unit weight of enzyme protein under specified conditions, there is no general agreement about the best definition and thus the specific activity may be expressed in various ways. As all enzymes are proteins it is common practice to relate the activity of an enzyme preparation simply to the amount of protein present. This may however be misleading, particularly when estimations of protein are based on methods such as the Folin-Ciocalteau (Lowry et al, 1951) due to the fact that this method relies on the presence of tryptophane and tyrosine in the protein and that consequently wide variations with different proteins may occur. In this respect the biuret method (Green & Cori, 1943) is preferable as the reaction is based on the peptide bonds within the protein molecule. This method was however found to be insufficiently sensitive for dilute purified preparations. In view of the fact that all three cellulolytic components of C.acremonium were found to be associated with carbohydrate at all stages of their purification, a feature common to other cellulolytic micro-organisms(Jermyn, 1962; Eriksson & Petersson, 1968; Petersson & Eaker, 1968; Norkrans, 1967; Selby & Maitland, 1967), specific activities were also calculated on the basis of total organic material as estimated by the method of Halliwell (1965). It must be noted that as both methods may also take into account the presence of any inactivated enzyme molecules it is essential that careful calculation of the extent of denaturation is made during purification procedures and that the number of enzyme

Specific Activities. (Table 10).

Purified Enzyme	Units/mg Protein (Lowry)	Units/mg Total Organic Material
β-glucosidase	2250	170
CMC-ase	1250	140
Cx-cellulase	1250 Cx-cellulase	100 Cx-cellulase
	600 CMC-ase	60 CMC-ase
	400 Cellulase	30 Cellulase

units be corrected by this figure before being quoted as the specific activity.

The results obtained indicated a very large contamination of protein by carbohydrate material in all cases of the order of approximately 10:1. While it was originally hoped to provide quantitative assessment of the purification acheived at various stages this did not prove to be possible in view of the high specific activities of the enzymes and the low concentrations of protein involved.

DISCUSSION

The study of microbial cellulase systems has now progressed to a stage where interest centres on the type of enzyme that must be synthesized by organisms capable of degrading unmodified native cellulose. In 1950 the existence of such an enzyme, C1, was postulated by Reese, Siu and Levinson who proposed that only those organisms possessing this enzyme should be classified as cellulolytic. Organisms incapable of attacking native cellulose were consequently termed non-cellulolytic despite any ability of these types to degrade modified cellulosic substrates by virtue of their Cx enzyme components.

Despite widespread acceptance of the basic C1-Cx concept, the resultant rigid classification of cellulolytic micro-organisms was subsequently ignored by numerous researchers (Basu & Pal, 1956; Norkrans, 1957a; 1957b; Nisizawa et al, 1961; Miller & Birzgalis, 1962; Petersson & Porath, 1963; Leatherwood, 1965; Logan & Siehr, 1966; Carpenter & Barnett, 1967; Ahlgren et al, 1967; Berg et al, 1968; Almin & Eriksson, 1968; Davies & Maclachlan, 1969; Patni & Rege, 1969) who continued to apply the terms cellulase and cellulolytic casually to enzymes and organisms on the basis of observations of CMC or swollen cellulose degradation. Several factors are believed to have contributed to such apparently unjustifiable use of this terminology. Prior to 1950 it was generally assumed that the enzymes attacking modified or substituted substrates were also responsible for the dissolution of native cellulose. Although Reese and his colleagues claimed that this was not the case, the validity of the postulated action of C1 in releasing short polyanhydroglucose chains from native

cellulose has more recently been seriously questioned as described earlier in this report. There is now increasing evidence (Halliwell & Riaz, 1971) that the Cx components may in fact be responsible for the comminution of fibrous cotton as envisaged prior to 1950, the probable action of C1 being to increase the accessibility of the fibres without producing any solubilization. Many workers in this field however undoubtedly confined their attentions to modified substrates purely on the basis of the simplified experimental procedures involved and not as a result of careful consideration of theoretical and experimental evidence. The use of an acid swollen cellulose substrate in the initial screening of cultures of Cephalosporia was consequently felt to be acceptable if the ability of the selected organisms to degrade native cellulose was also subsequently examined. Two organisms, C.recifei and C.acremonium, culture codes C25 and C123 respectively which were found to actively solubilize this acid swollen substrate were further investigated and shown to produce extensive though incomplete degradation of native cotton fibres in shake flask cultures. Although the existence of residual short fibres in such cultures was noted during these early investigations the significance of this observation was not fully realized at that time. Further early evidence of degradation of unmodified cellulose was provided by observations of rapid loss of tensile strength of unmercerized cotton by viable cultures using the apparatus shown in figure 27. Although measurements of loss of strength were found to be insufficiently accurate for screening cultures (the original purpose of the apparatus), both C.recifei and C.acremonium produced cleavage within 6 days incubation at 25°. In view of the existence of a C1 component implied by these

Figure 27.

Apparatus used for screening cultures for cellulolytic activity by measuring destruction of tensile strength of cotton thread.

a) Supporting beam.

b) Double wire hook sealed by epoxy resin.

c) Oxoid cap.

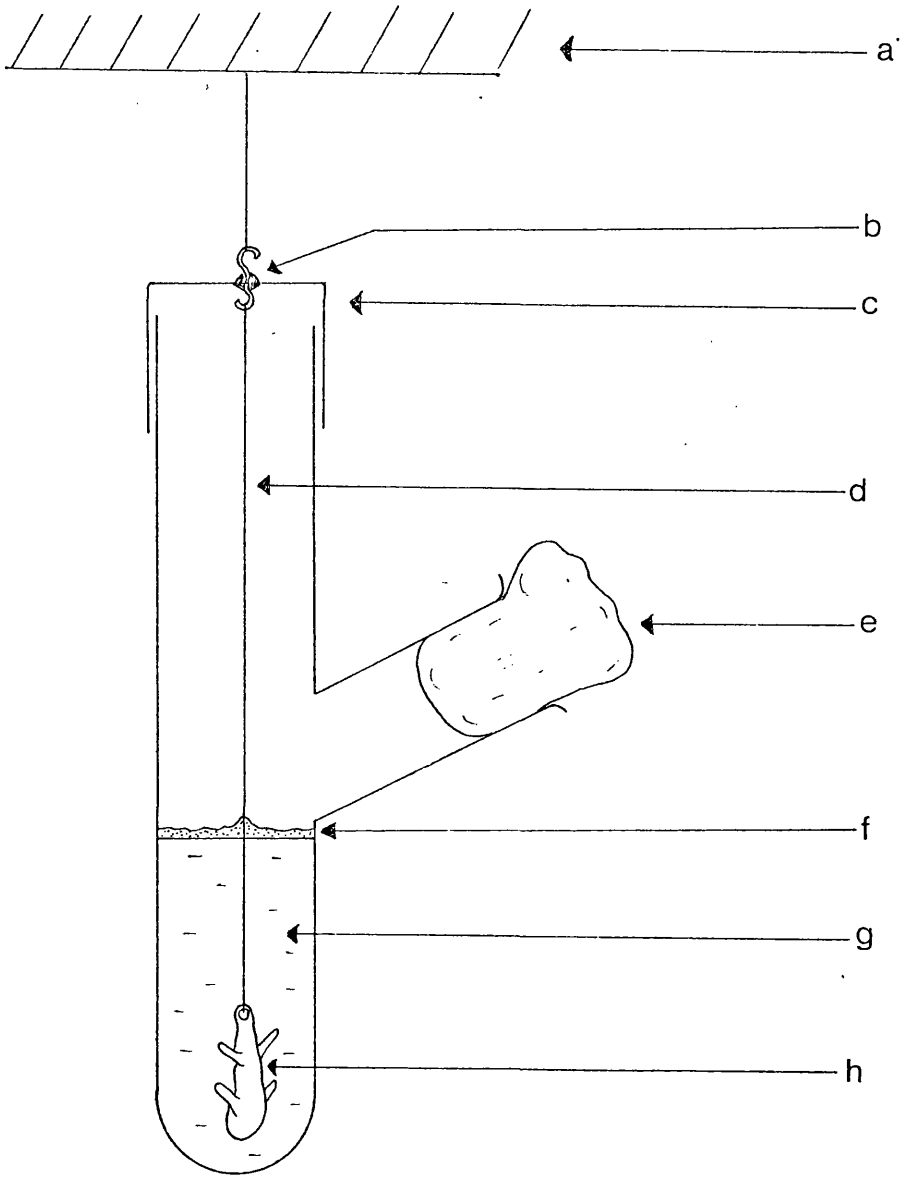
d) Cotton thread.

e) Inoculation port.

f) Mycelial mat.

g) Skinner's (1960) medium solidified with 2.0% w/v agar but lacking cellulose substrate.

h) Glass anchor.


a
b
c
d
e
f
g
h

observations, a detailed examination of the cellulase system of C.acremonium was initiated.

Preliminary studies demonstrated that in common with most cellulolytic fungi, growth was sustained by a wide variety of substrates with the notable exception of CMC. In spite of the poor growth of C.acremonium in cultures containing this substrate, cellulolytic enzymes were detected suggesting that although the preparation of CMC used was susceptible to enzymic hydrolysis, the substituted soluble products released by this process were not utilized intracellularly. The importance of degree of substitution in respect to the biodegradability of CMC preparations has already been discussed in the results section of this report.

Until comparatively recently, the synthesis of fungal cellulases was believed to be induced only by cellulose, soluble β-1,4 glucans or short chain oligosaccharides (Mandels & Reese, 1957; Mandels & Reese, 1960; Van Parijs, 1961). It is now generally accepted however that unlike most enzymes for which the inducers are usually the substrates, in polysaccharase formation it is the soluble products of hydrolysis which are the true inducers. Of the wide possible variety of hydrolytic products formed from cellulose, cellobiose has been regarded by many as the primary inducer of cellulolytic enzymes (Mandels & Reese, 1960; Norkrans, 1957a) although its role in this respect is complex. In addition to inducing synthesis, cellobiose has been reported as acting as an inhibitor of both cellulase formation (Mandels & Reese, 1960; Mandels & Reese, 1962; Norkrans, 1957a) and action (Reese, Gilligan & Norkrans, 1952; Norkrans, 1957a). In the case of the former, cellobiose concentrations of 0.5% to 1.0% were found to strongly repress cellulase formation

although this effect could also be produced by other rapidly metabolized carbon sources such as glucose or glycerol. There is also evidence that these same sugars added at 0.5% to 1.0% to a culture producing cellulase on cellulose may inactivate the enzymes already formed, with the reappearance of activity after consumption of the sugar (Mandels & Reese, 1960). This observation could be explained in terms of product inhibition in the cases of cellobiose and glucose, but not in the case of glycerol. In contrast to these results, cellobiose at a concentration of 1.0% was found to be an efficient inducer of Cx-cellulase, CMC-ase and particularly β-glucosidase in cultures of C.acremonium.

One of the most intriguing problems in cellulase research is the process by which an insoluble macromolecular substrate can induce a fungus to synthesize cellulolytic enzymes. The most common explanation implies that adaptive or inducible enzymes are not synthesized de novo on induction, but are constantly being produced in extremely small quantities;- too small to be detected by current analytical techniques. On contact with the substrate these few enzymes liberate minute amounts of soluble products which can enter the cell and stimulate the organism to increase the rate of enzyme synthesis. In the case of cellulolytic enzymes, if cellobiose is the inducer then growth of the organism on this substrate should give yields of cellulase comparable to those obtained with cellulosic substrates. Contrary to this hypothesis, titres of Cx enzymes were found to be eventually greater in cultures of C.acremonium containing cellulose than in those containing cellobiose, and Mandels & Reese (1960) demonstrated that most common cellulolytic fungi including T.viride

yielded only traces of cellulase when grown on the disaccharide. This observation was confirmed in the case of cellobiose-grown cultures of C.acremonium . By substituting cellobiose octaacetate however, Mandels & Reese (1960) obtained enzyme titres similar to those measured using cellulose and concluded that a slow gradual release of small amounts of cellobiose was essential for induction. A similar effect could also be produced in T.viride by a general reduction in metabolic rate by imposing conditions of suboptimum temperature and aeration or mineral deficiency etc.

The majority of reports implicating cellobiose as inducer have however apparently disregarded the fact that many microbial cellulase systems contain a β-glucosidase component, and the possible role of glucose as an inducer has not until recently been adequately investigated. Glucose may be considered to invoke an inducer-repressor mechanism of regulation of enzyme synthesis when added to cellulose as it represses the induction of cellulase unless present in minute amounts (Horton & Keen, 1966). These workers showed that cellulase formation in the plant pathogen Pyrenochaeta terrestris was repressed to the basal level by 5×10^{-4} molar glucose. In view of the fact that glucose concentrations in excess of this figure are normally present in plant cells, these workers believed cellulase action to be relatively unimportant in the primary attack, primary invasion being more a result of polygalacturonase activity. In the presence of β-glucosidase the production of cellulolytic enzymes would not be expected to be repressed as the metabolism of the organism would be limited by the relatively slow liberation of glucose from cellulose. Hulme and Stranks (1970, 1971) investigated the

effect of supplying laboratory cultures with glucose at a rate comparable with its release from cellulose, and observed that production of cellulase was controlled by the rate of metabolism when regulated by carbohydrate uptake. In addition these workers noted that while cellulolytic enzymes could be detected at all times during incubation of shake flask cultures of M.verrucaria containing glucose as sole carbon source, a marked increase in the titre of the enzymes was noted as substrate exhaustion became imminent. These observations compare closely with those made on C.acremonium although release of cell bound enzymes may have contributed to this increase as a result of cell lysis during the senescent phase of growth of this organism. Hulme and Stranks (1970,1971) also demonstrated that this phase of imminent glucose exhaustion could be sustained by means of a small scale fermentation apparatus, enabling more constant conditions to be maintained. These studies clearly established that other carbon sources such as glycerol and glycine could replace glucose without affecting cellulase production, and that a variety of other polysaccharases were synthesized simultaneously with cellulase under these conditions. The latter observation is of considerable importance in polymerase induction theories which have never adequately explained how an organism recognizes the polymer from which the inducing soluble products are derived. Hulme and Stranks' work indicates that no such recognition apparently occurs; the mild stress conditions produced by limited carbohydrate supply induce the organism to increase the rate of replication of all the types of polysaccharases capable of being synthesized by that organism. This theory would explain previous observations of xylanase, mannanase and aryl-β-gluco-

sidase induction by cellulose (Mandels & Reese, 1962; Eriksson & Rzedowski, 1969) and also account for the powerful cellulase inducing action of the slowly metabolizable sophorose (β-1,2-glucoside) described by Norkrans (1967) and of lactose on T.viride (Mandels & Weber, 1969).

As noted earlier, qualitative examinations had established C.acremonium to be capable of hydrolysing native cellulosic substrates with the consequent implication of C1 enzyme production by this organism. More detailed quantitative studies of shake flask cultures demonstrated however that only approximately 50% of such native substrates were apparently susceptible to attack by C.acremonium, and the subsequent observation that the residual refractory short fibres were rapidly hydrolysed by cultures of T.viride but not by fresh cultures of C.acremonium seriously questioned the synthesis of a C1 component by the Cephalosporium. Because the original purpose of this investigation was to elucidate the cellulase system of a truly cellulolytic micro-organism which had not previously been examined and to study the mode of action of the C1 component, a decision regarding the continued investigation of C.acremonium became necessary at this stage. The decision to continue with the investigation was based on the proposal that any organism capable of producing such extensive degradation of native cellulose without the assistance of a C1 component must possess a unique cellulase enzyme system, or be in an intermediate position between the previously rigidly separated groups of cellulolytic (possessing C1) and non-cellulolytic (lacking C1) organisms.

The isolation and purification of the enzymic components of C.acremonium cellulase system were only achieved by careful control

of fractionation conditions. In contrast to fractionations of extracts from F.solani (Wood, 1971) on Sephadex G100 and from Polyporus versicolor (Petersson et al, 1963), M.verrucaria (Selby & Maitland, 1965) and a species of Aspergillus (Ahlgren et al, 1967) on Sephadex G75, all C.acremonium components were excluded from these gels as a result of their greater molecular dimensions. In this respect extracts of C.acremonium exhibited some similarities to those of T.viride (Selby & Maitland, 1967) and T.koningii (Wood, 1968), although both of these organisms were characterized by the separation of a second low molecular weight CMC-ase component on Sephadex G75 which was absent from the Cephalosporium extracts. Such a component was however demonstrated in Cephalosporium extracts during fractionation on Sephadex G200. This was consequently of interest when it was noted that this second peak of CMC-ase was closely associated with the Cx-cellulase and cellulase activities of the extract. Because Selby and Maitland and Wood only made use of CMC to estimate Cx enzyme activities, no information is available as to whether their low molecular weight CMC-ase components possessed any activity towards other modified substrates. The apparent lack of foresight of these workers in failing to screen their fractions against an accessible insoluble cellulose preparation in addition to CMC is surprizing as it is well known that the use of CMC alone can lead to the omission during fractionation studies of important components of the cellulase complex. As early as 1954 Gilligan & Reese described an enzyme from M.verrucaria which was only slightly active towards CMC but which was capable of rapidly hydrolysing Walseth cellulose. Conversely Wood (1969) has reported that certain fungal preparations rich in CMC-ase

activity were inactive on Walseth cellulose, and it is interesting to note that this worker has more recently included both types of substrate in fractionation studies of F. solani (Wood, 1971). Although it is thus not possible as yet to implicate the low molecular weight CMC-ase components of T.viride and T.koningii with any other Cx function, the action of these enzymes towards native cellulose is known. While the T.viride component was shown to be inactive against cotton fibres, Wood established the T.koningii component to be capable of producing slight solubilization of cotton and to make an appreciable contribution to the total S-factor activity of unfractionated extracts. Both components were however similar in that neither appeared to be essential enzymes of the cellulase systems of these organisms. The situation in C.acremonium extracts was however markedly different, the low molecular weight CMC-ase component in this case being shown to be closely associated with the total Cx-cellulase and cellulase activities of the extract. Subsequent fractionations were consequently directed primarily towards the further resolution of these activities.

Although Selby & Maitland (1967) and Wood (1968) established that the C1 components of T.viride and T.koningii were strongly adsorbed on DEAE-Sephadex while the bulk of the Cx-enzymes were rapidly eluted, this ion exchange material was unfortunately unavailable during the early stages of C.acremonium fractionations to establish the presence of a C1 enzyme. When however DEAE-Sephadex became available at a later date, C1 activity could not be demonstrated in the Cephalosporium system, and chromatography on DEAE-Sephadex did not achieve a separation of cellulase, Cx-cellulase and low molecular weight CMC-ase components. In addition

to this chromatographic evidence, results of other analytical work pointed to the fact that a single enzyme species was responsible for all these activities in C.acremonium extracts. This evidence may be summarised briefly as:

a) Samples of filtrates from shake flask cultures which contained CMC-ase and Cx-cellulase but which were apparently devoid of cellulase could be shown to be active against cotton fibres after being concentrated 10-fold by freeze drying.

b) No further resolution of these activities was achieved on Sephadex G200 when the K15/90 and K25/100 chromatography columns were connected in series by means of a flow adaptor despite an effective doubling of bed height by this modification.

c) Purified samples containing these activities exhibited an approximate ratio of low molecular weight CMC-ase : Cx-cellulase : cellulase of 2.5:3:1. This ratio remained essentially unchanged throughout subsequent fractionation and denaturation studies.

d) All three activities exhibited similar temperature and pH profiles.

e) Denaturation rates of Cx-cellulase and cellulase in crude filtrates stored at 4^{o} and recoveries of these activities after fractionation were found to be similar.

f) All three activities were associated with a single peak following preparative discontinuous gel electrophoresis, this peak being subsequently shown to migrate as a single protein band when refractionated on small scale disc electrophoresis gels at pH 9.5. Although further attempts were made to demonstrate homogeneity by gel electrophoresis at a variety

of gel pH values, polymerization problems were encountered which prevented such studies being undertaken. In view of the powerful resolution of discontinuous electrophoresis however, the migration of the low molecular weight CMC-ase, Cx-cellulase and cellulase activities as a single protein band at pH 9.5 was believed to be highly indicative of a single enzyme species.

These results strongly suggested that the cell-free cellulase system of C.acremonium consisted of three individual components; an extremely high molecular weight β-glucosidase, a CMC-ase and a lower molecular weight enzyme exhibiting little substrate specificity, being capable of producing extensive degradation of CMC and acid swollen cellulose and more limited solubilization of native cotton fibres. The term Cx-cellulase was consequently retained for this component.

Concurrent examinations and fractionations of solubilized cell-bound cellulolytic enzymes revealed the presence of three components possessing similar features to those isolated from cell-free extracts. Numerous investigations have been carried out into methods for solubilizing proteins bound to cellular structures (Schneiderman, 1965; Mitchell & Hanahan, 1966), cell bound proteolytic enzymes (Norberg & Hofsten, 1969) and other depolymerases including cellulase (Barash et al, 1969; Porter, 1970; Yamane et al, 1970a, 1970b), the treatments required for effective solubilization ranging in intensity from contact with buffer at 4° (Barash et al, 1969) to the use of high concentrations of urea and mercaptoethanol (Schneiderman, 1965) or sonication (Yamane et al, 1970). As a result of kinetic studies on cell-bound and cell-free ascorbate oxidase preparations, Hallaway et al

(1970) concluded that there were three possible explanations for the existence of identical enzymes in both cell-bound and cell-free extracts of an organism. Thus the enzyme may be normally present on both sites, or it may be normally cell-bound and is released during the preparation of cell-free extracts, or it may be normally cell-free and is absorbed on to cellular material during extract preparation. Hallaway and her co-workers pointed out that the latter case would be unlikely to be operating if the bound enzymes subsequently required harsh treatment for their re-release, and it is interesting to note that cell-bound cellulase components of C.acremonium could be readily solubilized by extremely mild treatments. Although binding of an enzyme often results in a reduction of activity (Silman & Katchalski, 1966) it is reasonable to assume that such binding would be least inhibitory to an enzyme whose substrate is a soluble small molecule. In the case of a depolymerase acting on an insoluble substrate however, the loss of mobility resulting from binding might be expected to place severe restrictions on enzyme activity with the possible exception of enzymes bound to the external surfaces of fungal hyphae. In this case it is possible to envisage the transportation of the depolymerase by hyphal penetration into substrate interstices which might be inaccessible to soluble enzymes capable only of diffusion (Hubert, 1924; Bailey & Vestal, 1937; Norkrans, 1957; Duncan, 1960). It would thus be possible on the basis of this theory to predict that the greatest increase in activity of cell-bound C.acremonium cellulase components would be exhibited by the Cx-cellulase on solubilization and the smallest increase by β-glucosidase; predictions which as noted earlier were confirmed experimentally.

Further evidence to support this theory is provided by the observation that cell-bound cellulase activity which was apparently due to Cx-cellulase action was only detected after solubilization treatments were applied to cell fragments.

Although these results do not indicate which of the mechanisms of enzyme binding proposed by Hallaway et al (1970) may be operating in C.acremonium, it is possible to theorize about the purpose, if any, of such components. The specialized example of hyphal-bound depolymerases has already been noted, but a more frequently encountered situation is that in which multi-enzyme systems are structurally organized to increase the overall rate of reaction. Although the loss of enzyme mobility due to binding might initially be considered to give rise to a reduction of reaction rate, the reduced distance between enzyme molecules results in the transit time of reacting molecules being greatly diminished leading to a higher efficiency. This organization may be developed to such an extent that topographical arrangement may occur (Dixon & Webb, 1960), imposing a further control on the reaction. Although this organization is usually associated with complex enzyme systems involved in oxidative metabolism, and particularly in the case of mitochondria and chloroplasts, a multienzymic depolymerase system could theoretically benefit from such a spatial arrangement by virtue of the low transit times involved and the lack of possibility of product escape. A relatively low reaction rate may be expected in soluble enzyme systems involving high molecular weight intermediates, the situation existing in most depolymerase systems and particularly in the case of cellulase enzymes.

As noted earlier, C.acremonium is apparently unique in

being capable of producing extensive degradation of native cellulose without the aid of a C1 component, and it is possible to conjecture that this activity may be due to some specific organization of Cx components on the external mycelial surfaces of this organism. In order to elucidate whether any such orientation of enzyme sites exists, highly specific labelling techniques would probably be required. Most techniques based on enzyme specificity and the labelling of released products are unlikely to be sufficiently specific in the case of the C.acremonium cellulase complex due to the overlapping action of the component enzymes. In this case an interesting line of research could be the development and study of labelled antibody-antigen reactions coupled with detailed electron microscope studies similar to those recently carried out by Smirnova et al (1971) on bacterial exo-enzymes and toxins.

Although the degradation of native cellulose by C.acremonium is extensive for an organism possessing only a Cx enzyme system, the component enzymes forming that system were found to be similar in many respects to other microbial cellulolytic enzymes. Thus the optimum conditions of pH and temperature correspond closely to those previously noted for F.solani (Wood, 1969), T.koningii (Wood, 1968), M.verrucaria (Halliwell, 1964), Chrysosporum lignorum (Eriksson & Rzedowski, 1969), Collybia vellutipes (Norkrans, 1957) and T.viride (Selby & Maitland, 1967), although the C.acremonium components were generally active over a wider pH range than for most of these organisms. A characteristic feature of fungal cellulase systems would seem to be the much higher temperature (37°-45°) required for optimum enzyme activity than for optimum growth of the organism (25°-30°), indicating

that cellulase enzymes generally possess greater thermostability than some other metabolically essential enzyme(s). This was exemplified by the extreme thermostability of the C.acremonium β-glucosidase component which was also the largest molecule of the cellulase complex and might thus be thought to be the most susceptible to denaturation by disruption of the tertiary structure of the molecule. While this is believed to indicate a high degree of cross linkage between the folded protein chains of the β-glucosidase molecule, the nature of the linkages involved is as yet unknown.

Since it is well known (Dixon & Webb, 1960) that the tertiary structure of proteins and their enzyme activity are related, it may well be that combination of enzymes with their large molecular weight substrates as in the case of cellulases may contribute to the apparent thermostability of these enzymes. In this work all C.acremonium components were found to be associated with appreciable quantities of carbohydrate which were not removed by any of the fractionation techniques utilized. This again appears to be a common characteristic feature of Cx enzymes as Norkrans (1967) noted that no completely carbohydrate-free Cx enzyme had been isolated upto that time. The association of carbohydrate with purified enzymes does not seem to be limited to the Cx types as Selby & Maitland (1967) demonstrated the C1 component of T.viride to be a glycoprotein with a carbohydrate:protein ratio of 1:1. Although this component was homogeneous on Sephadex G75, DEAE- and SE- Sephadex and migrated as a single protein band during disc electrophoresis, the possibility remains that the multiple Cx components isolated by a number of workers as described previously may be due to isoenzymes or to carbohydrates

complexing with the protein moieties of Cx. Thus Jermyn (1952) found that the β-glucosidase of Stachybotrys atra was dissociable from the carbohydrate component, and Eriksson & Petersson (1968) succeeded in separating the carbohydrate associated with two cellulolytic components of Stereum sanguinolentum, this purification rendering the two components indistinguishable by ion-exchange chromatography and electrophoresis. These results indicated that S.sanguinolentum liberated only a single cellulolytic component when grown on powdered cellulose, a theory subsequently confirmed by physico-chemical characterizations (Bjorndal & Eriksson, 1968). Petersson & Eaker (1968) also achieved separation of carbohydrate from a Cx cellulolytic enzyme from Penicillium notatum by chromatography on hydroxylapatite. Further study of this enzyme by Eriksson & Petersson (1968) demonstrated that although the enzyme remained active following removal of carbohydrate, the integrity of disulphide bridges between peptide chains was essential for activity by maintaining the tertiary structure of the molecule. It does however seem likely that complexing of substrate and enzyme contributes markedly to the thermostability exhibited by most cellulolytic enzyme systems.

In view of the widespread nature of such complexes and the lack of success in their separation by the majority of workers, most of the molecular weight estimations carried out on cellulase components must inevitably be in excess of the pure enzymes' true dimensions. Nevertheless, an extremely wide variation of molecular weights has been reported for the various components of microbial cellulases, varying from 5,300 for a component of M.verrucaria (Selby & Maitland, 1965) active against cotton to 400,000 in the

case of F.solani β-glucosidase (Wood, 1971). This demonstrates another apparently characteristic feature of cellulases and other typically extracellular endoenzymes such as mannanase and xylanase that these enzymes are generally found to be smaller than the corresponding exoenzymes such as β-glucosidase, mannosidase and xylosidase (Petersson & Porath, 1963; Ahlgren et al, 1967; Eriksson & Rzedowski, 1969). For the former group insoluble polysaccharides are the normal substrates requiring small enzyme molecules for efficient substrate penetration, whereas the second group acts preferentially on soluble substrates. Thus as might be predicted on the basis of this inverse relationship, most of the Cx enzymes examined so far have exhibited apparent molecular weights of about 50,000 (Whitaker, Colvin & Cook, 1954; Petersson & Porath, 1963; King, 1966; Selby & Maitland, 1965; Norkrans, 1967), and the cellulase system of C.acremonium shows no deviation from this pattern, estimates of molecular weights being approximately 45,000 for the CMC-ase and in excess of 200,000 for the β-glucosidase. The estimated molecular weight of approximately 11,000 for the Cx-cellulase component is rather unique for such a Cx enzyme although Petersson & Porath (1963) have isolated an enzyme of similar dimensions from Polypor us versicolor. Unlike the Cx-cellulase component of C.acremonium however, this enzyme was not shown to be capable of attack on native cellulose. Although the C1 component might thus be expected to be a molecule of extremely small dimensions, Selby & Maitland (1967) estimated that this component in T.viride possessed a molecular weight of approximately 61,000. Although this is an apparent contradiction of the general size distribution of cellulase components it must be remembered that in all of the foregoing work estimates of

molecular weight have been made on samples of enzymes which had not been previously separated from complexed carbohydrate. An indication of the extent to which such estimates may thus be in error is provided by the work of King (1966) and Li *et al* (1965) who estimated the molecular weight of an endoglucanase to be approximately 52,000 by sedimentation data but of only 26,000 by amino acid analysis. This latter value corresponds closely to the molecular weight estimates for the Cx enzyme of *Stereum sanguinolentum* obtained by Bjorndal & Eriksson (1968) following successful separation of the enzyme-carbohydrate complex. Thus the C1 of *T.viride* may in reality be a protein molecule of much smaller dimensions than that envisaged by Selby & Maitland (1967). Similarly, separation of carbohydrate from the Cx-cellulase of *C.acremonium* could reveal an extremely low molecular weight enzyme which may explain the ability of this component to attack not only swollen accessible forms of cellulose but also the more permeable regions of native cotton fibres. In the case of *M.verrucaria* (Selby & Maitland, 1965) the low molecular weight (5,300) component acted on cotton in conjunction with another component of molecular weight about 55,000. Although the small enzyme was adsorbed by cotton fibres, no hydrolysis was apparently possible by this component in isolation. As the Cx-cellulase of *C.acremonium* was also strongly adsorbed by cotton but was in addition capable of attacking this substrate under suitable conditions of temperature and pH, there would seem to be an extremely fine dividing line between the modes of action of these two enzymes, one of which is capable of completely degrading cotton fibres to soluble products while the other is more restricted in its action.

More information on the accessibility of cellulose to molecules of varying dimensions has been provided by Stone *et al*, (1969) who suggested the most likely size for a Cx enzyme molecule to be approximately 30-40 Å in diameter. Whitaker *et al* (1954) had earlier reached the conclusion that such an enzyme from *M.verrucaria* with a molecular weight of 60,000 was ellipsoidal in solution with a length of 200 Å and a diameter at its centre of 33 Å. This was in agreement with Stone's calculations and apparently suggested an endwise penetration of the enzyme into the substrate molecule. Although this may seem inherently unlikely, Stone has pointed out that the pores in swollen cellulose probably exist as slit-like spaces and that molecular sieves frequently separate ellipsoidal molecules according to their minor axes. This work thus demonstrates not only the advantages possessed by an enzyme molecule of small dimensions but also re-affirms the importance of the porosity of the substrate in addition to its degree of crystallinity. The considerable limitations placed on the activity of *C.acremonium* Cx-cellulase by the lack of porosity and the high degree of crystallinity in cotton fibres was reflected in the observation that enzyme saturation could not be easily achieved by this substrate.

Both the CMC-ase and Cx-cellulase components of *C.acremonium* are suggested as being endoenzymic in nature by the results obtained from paper and column chromatography and viscometry studies. As noted earlier, Reese (1969) has proposed a new method for confirming the mode of action of cellulase components based on their relative activities towards cellotetraose and cellobiose. The method involves the estimation of glucose by glucose oxidase,

and although it was hoped that this technique could confirm the mode of attack by C.acremonium components it soon became apparent that the glucose oxidase preparation (BDH) itself contained appreciable hydrolytic activity towards cellobiose. This possible lack of specificity was not mentioned by Reese, and the method is thus invalid unless the homogeneity of the preparation has been previously confirmed. There would seem to be no reason against the use of alternative enzymic analyses for glucose such as hexokinase in order to relieve such problems. In the case of the β-glucosidase component it is not possible on the basis of the analyses carried out to state whether this is an endo- or exo-enzyme, although the results of viscometric studies would suggest the former.

In view of the broad substrate specificity exhibited by the Cx-cellulase component of C.acremonium it is difficult to offer an explanation for the synthesis of an apparently superfluous CMC-ase enzyme by this organism. Although the CMC-ase component was found to exhibit a greater affinity for CMC than the Cx-cellulase, the degradation of this substituted substrate by the latter is quite rapid and occurs in a similar manner to that brought about by the CMC-ase. Although CMC is not a natural substrate, the manner and extent to which it is attacked provides an insight into the process of hydrolysis of soluble intermediates released from native cellulose, and it is therefore unlikely that the loss of the CMC-ase component would be detrimental to the organism's ability to attack cellulose. This phenomenon has been previously noted in the case of T.viride (Selby & Maitland, 1967) and T.koningii (Wood, 1968) where low molecular weight CMC-ase components had no effect on the

organisms' capacities to solubilize cotton. In both of these cases however a higher molecular weight CMC-ase was an essential component of the cellulase complex. C.acremonium is consequently believed to be unique in possessing a single enzyme capable of extensive attack on a variety of cellulosic substrates ranging in complexity from native cotton fibres to cellotriose. In retrospect it would have been interesting to determine the Michaelis constants for the action of Cx-cellulase on cellotriose and cellotetraose. Although the Cx-cellulase is inactive towards cellobiose, the cellulase complex is completed by a β-glucosidase component capable of rapidly hydrolysing the disaccharide to glucose. It is interesting to note however that while almost all the S-factor activity of C.acremonium filtrates was subsequently found to be due to Cx-cellulase action, the CMC-ase component was capable of producing quite significant losses of tensile strength in cotton fibres without any simultaneous measurable solubilization. Conversely, Wood (1968) noted that the low molecular weight CMC-ase of T.koningii solubilized cotton to a very limited extent but made an appreciable contribution to the overall S-factor activity.

How adequate then are the components of the cellulase system of C.acremonium, and how well organized to enable this organism to degrade native cellulosic materials? The possibility of some structural arrangements of enzyme molecules on the penetrating hyphal strands has already been discussed, and it is interesting to note the microscopic changes occuring in cotton fibres during their attack by viable cultures of C.acremonium. The most distinctive morphological change in such fibres is that of increasing lumen size (plates 3-5), a phenomenon which is in

close agreement with observations made by other workers that fungal attack on cotton usually involves the growth of hyphae within the lumen. This is accompanied by complete or partial dissolution of wall material from within, with transverse cracking and spiral fissures visible at the early stages (Norkrans, 1967). This is in contrast to bacterial attack which is usually initiated from the outer surface (Siu, 1950). In studies on the destruction of hair by Chrysosporium keratinophylum, English (1969) found that this organism primarily attacked the lumen by penetrating the fibres with boring hyphae and "fronded" mycelium. This physical penetration was followed subsequently by enzymic hydrolysis with the hair fibre being gradually replaced by mycelium. It is thus possible to draw an analogy between the destruction of hair by this organism and the degradation of cotton fibres by cellulolytic micro-organisms although it should be noted that in the latter case no specialized cellulose eroding organs have been observed. Norkrans (1967) has confirmed the considerable mechanical forces involved in hyphal penetration of substrates in the case of the blue-staining fungi whose club shaped hyphal heads are provided with spear-like projections which are capable of penetrating metal foils.

In the case of C.acremonium however the precise mode and site of action of the Cx-cellulase component on cotton fibres remains uncertain. This attack is characterized by the fragmentation of the fibre into numerous short fibres which are apparently resistant to further Cx action. Although fibre fragmentation has been previously reported as a preliminary stage in the degradation of cotton (Halliwell, 1965) this was found to be rapidly followed by solubilization of the small fibres in the

case of T.koningii. The only other report of a micro-organism attacking cotton in a similar manner to C.acremonium appears to be that quoted by Norkrans (1967) of a brown-rot fungus Poria monticola, which leaves cellulose entities of about crystallite size unattacked. The residual short fibres observed in cultures of C.acremonium are however of considerably greater magnitude, and more closely resemble those liberated by T.koningii which were up to 3mm in length (Halliwell, 1965). Although cell-free extracts of C.acremonium were capable of liberating soluble sugars from native cotton, short fibres were only produced in the presence of the viable organism. Such restricted solubilization has been previously reported by Selby, Maitland & Thompson (1963) using extracts from M.verrucaria; these workers attributing the low activity to localised attack on the cellulose and to enzyme immobility in crystalline regions of the substrate. If this is the case with C.acremonium, the residual short fibres would be expected to possess a higher average degree of crystallinity than the original cotton. Such an increase in relative crystallinity has been previously noted several times (Norkrans, 1950a, 1950b; Walseth, 1952; Kaplan et al, 1970). With the facilities available for this investigation however, X-ray crystallography failed to produce any evidence of increased crystallinity in the residual fibres although refractive index studies suggested that such an increase had occurred.

This pattern of degradation may consequently be believed to indicate enzymic attack by Cx-cellulase on isolated areas of the cotton fibre, and that these areas consist mainly of more accessible and consequently more reactive amorphous cellulose. Some confusion now exists however in equating accessibility of

cellulose with its amorphous fraction, and Stone et al (1969) are of the opinion that any correlation found between the amount of amorphous cellulose in a sample and its susceptibility to attack by cellulase is coincidental despite the fact that as cotton is decrystallized it becomes increasingly reactive (Reese, Segal & Tripp, 1957). Rautela & King (1968) however studied the induction and action of cellulases on a variety of crystal types of cellulose and found that the crystal structure as well as the crystallinity of the cellulose remained largely unchanged during its dissolution. It is interesting to note that these workers also observed fibre fragmentation by electron microscopy and found that the enzymic degradation of cellulose involved fragmentation which appeared to be initiated by the formation of longtitudinal fissures. Stone et al (1969) believe however that the porosity of the substrate is the most important factor governing subsequent enzymic attack, and it may be possible that the degradation of cotton by the Cx-cellulase of C.acremonium occurs at areas consisting of highly porous but crystalline material. The analytical techniques used to establish any increase in degree of crystallinity following degradation in this investigation were unfortunately insufficiently sensitive to ascertain which type of material had undergone attack. Although this is a point which requires clarification it seems almost certain that the sites of attack are areas of cellulose which are sufficiently accessible to allow ingress and diffusion of the relatively small Cx-cellulase molecules. If such areas are numerous and distributed randomly throughout the fibre, attack at these sites would account for the rapid overall loss of tensile strength and the eventual fragmentation of the fibre.

The soluble polymeric sugars released by the action of the Cx-cellulase could then be depolymerized by this same enzyme or by the CMC-ase component to yield short chain oligosaccharides and cellobiose on which β-glucosidase is predominantly active. It will be noted that this extensive degradation occurs without the aid of a C1 enzyme, and more recently Halliwell and Riaz (1971) have shown that the short fibre forming activity of T.koningii filtrates is not associated with the C1 component of this organism but is due to a separate enzyme species which these workers termed C2. In addition to these two enzymes, T.koningii filtrates also contained CMC-ase and cellobiase enzymes. By disregarding the C1 enzyme considerable similarities between the enzyme systems of T.koningii and C.acremonium thus become apparent, particularly in the case of the C2 and Cx-cellulase enzymes. As noted earlier, Wood (1968) suggested that in view of the similarities exhibited by the cellulase systems of T.viride (Selby & Maitland, 1967), T.koningii (Wood, 1968) and F.solani (Wood, 1969) it was possible that there existed a fundamental mechanism for the degradation of native cellulose rather than a random mixture of enzymes. The cellulase enzyme system of C.acremonium has thus been shown to conform closely to this mechanism in many respects, although attempts to demonstrate cross-synergism between isolated components of C.acremonium and those of the other organisms were not made.

C.acremonium remains the "odd man out" of the organisms described above in that it lacks a C1 enzyme, and it is an intruiging thought that the Cephalosporium may be an organism in the upward evolutionary process of evolving a complete spectrum of cellulolytic components.

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ERRATA

1) Page 31 line 14; for "Selby & Maitland 1967" substitute "Selby 1969".

2) Page 35 line 22; for "90% v/v" substitute " approximately 9.0 molar".

3) Page 35 line 25; for "15 lb pressure" substitute "15 psi".

4) Page 38 line 15; for "... at 0°" substitute "... at 0° for 30 min".

5) Page 39 line 6; for "citrate buffer" substitute "sodium citrate buffer".

6) Page 41 line 24; for "4,000 rpm" subsitiute " approximately 2,000 g".

7) Page 43 line 16; for "4,000 rpm" substitute " approximately 2,000 g".

8) Page 49 lines 12, 14, 15, 16; for "acetate" substitute " McIlvaine ".

9) Page 50 line 20; for "3,500 rpm" substitute "approximately 1800 g".

10) Page 51 line 25; for " (4:1) " substitute " (4:1 v/v) ".

11) Page 51 line 27; for "phosphoric acid" substitute "ortho-phosphoric acid".

12) Facing fig. 3 lines 5 & 8; for "gluconolactone" read "1.0 mg/ml gluconolactone".